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# Pathogenesis and Etiology of Congenital Diaphragmatic Hernia In an Animal Model

by ©

Randal Peter Babiuk

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Physiology

Edmonton, Alberta

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November 27, 2002

**To my mother and my entire family:**

**I couldn't have done it without your love and support.**

**Thank you**

# ABSTRACT

Congenital diaphragmatic hernia (CDH) is a serious birth defect that is manifested by a hole in the diaphragm. There is high mortality and severe morbidity of affected infants. In this thesis, an animal model of congenital diaphragmatic hernia has been used to address several issues. The first project examined the embryonic origins of the diaphragm muscle. Immunohistochemical labeling for developmentally expressed markers of muscle development was utilized to identify the source of the diaphragmatic musculature. From these studies, the primary source of the diaphragm muscle was determined to be myogenic cells from cervical somites that migrated to the pleuroperitoneal fold (PPF).

Secondly, the hypothesis that the initial defect in CDH occurs in the mesenchymal substratum of the diaphragm was examined. *C-met* null mice were used to examine the nonmuscular component of the diaphragm and its role in the pathophysiology of CDH, and to test the hypothesis that a lung defect is the primary problem leading to development of CDH. It was found that hernias could be induced in the amuscular membrane using the same compounds that induced hernias in muscularized diaphragms. Also, in *FGF10* $^{-/-}$ mice with no lungs, a hernia could be induced in the diaphragm of a null fetus, demonstrating that CDH could occur in the absence of lungs.

The third project was an investigation of the hypothesis that the etiology of the CDH model involves the retinoid system. Four teratogens that independently induce diaphragmatic hernias in fetal rats were tested for the ability to inhibit retinoic acid

production. All four compounds significantly diminished or abolished retinal dehydrogenase activity, strongly suggesting a definite link between the retinoid system and the chemical model of CDH.

The results of the study of the normal and pathological development of the diaphragm have shed new light on the long-held ideas about diaphragm formation and the tissue origin of the defect in CDH. Specifically, major findings from this work provide insight into the following: *i*) the embryonic origin of the diaphragmatic musculature, *ii*) the pathogenesis of CDH in the animal model, and *iii*) the potential involvement of the retinoid system in CDH etiology.

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# LIST OF ABBREVIATIONS

The following abbreviations, definitions and units have been used throughout this thesis.

| | |
|---|---|
| ADH | alcohol dehydrogenase |
| CDH | congenital diaphragmatic hernia |
| CRABP | cellular retinoic acid binding protein |
| CRBP | cellular retinol binding protein |
| DAB | 3,3-diaminobenzidine tetra-Hydrochloride |
| DAPI | 4′, 6-Diamidino-2-phenyl-indol |
| DNA | deoxyribonucleic acid |
| DSHB | Developmental Studies Hybridoma Bank |
| DiI | 1,1'-dilinoleyl-3,3,3',3'-tetramethylindicarbocyanine perchlorate |
| E0 | embryonic day 0 |
| ECMO | extracorporeal membrane oxygenation |
| ENU | N-ethyl-N-nitrosourea |
| ET | endothelin |
| FBM | fetal breathing movements |
| GER | gastroesophageal reflux |
| GFP | green fluorescent protein |
| FGF-10 | fibroblast growth factor 10 |
| HFOV | high frequency oscillatory ventilation |
| HGF/SF | hepatocyte growth factor/scatter factor |
| HSLAS | Health Sciences Laboratory Animal Services |
| HRE | hormone response element |
| IGF-II | insulin-like growth factor-II |
| LRAT | lecithin:retinol acyl-transferase |
| MAb | monoclonal antibody |
| MCA | multiple congenital anomalies |
| MHC | myosin heavy chain |
| NO | nitric oxide |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PDGF-B | platelet-derived growth factor B |
| PPF | pleuroperitoneal fold |
| PPHN | primary pulmonary hypertension of the newborn |
| RA | retinoic acid |
| RALDH2 | retinal dehydrogenase-2 |
| RAR | retinoic acid receptor |
| RARE | retinoic acid response element |
| RBP | retinol binding protein |
| RNA | ribonucleic acid |
| RT | room temperature |
| RT-PCR | reverse transcription-polymerase chain reaction |
| RXR | retinoid X receptor |
| RXRE | retinoid X response element |
| SNHL | sensorineural hearing loss |
| SP-A,C | surfactant protein C |

| | |
|---|---|
| TRIS | tris[hydroxymethyl]-amino methane hydrochloride |
| TUNEL | TdT-mediated dUTP nick-end labeling |
| VA | vitamin A |
| VAD | vitamin A deficient |
| VDR | vitamin D receptor |
| WT1 | Wilms' Tumour Suppressor gene |

# CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

## *1.1 OBJECTIVES*

The main objectives of this work are *i*) to resolve the fundamental issue of the embryonic origins of the diaphragm, *ii*) to apply that knowledge to ascertain which aspect of diaphragm embryogenesis malforms in an animal model of congenital diaphragmatic hernia (CDH) and why this defect occurs and *iii*) to investigate the hypothesis that a defect in the retinoid system is the underlying etiology of CDH in the animal model.

Chapter 1 is a general introduction to provide the necessary background for an understanding of the research detailed in future chapters of the thesis. Included in the general introduction is information on the processes of diaphragmatic muscle development and the markers for certain stages of myogenesis that were utilized in the project upon which Chapter 3 is based. Also, a discussion of congenital diaphragmatic hernia in terms of the experimental and clinical aspects is provided for a basis on all the projects that make up this thesis work and has particular significance to Chapters 4, 5 and 6. The retinoid (Vitamin A and derivatives) system is a focus of the introduction and will give a framework to understand some material found in Chapter 5.

Chapter 2 is a methods section describing common techniques used in the individual projects, such as immunohistochemistry, tissue dissection and processing, and administration of chemicals to pregnant animals.

Chapter 3 addresses Objective *i* and describes work that indicates the muscle precursors that migrate to the primordial tissue structure from which the diaphragm forms, the pleuroperitoneal fold (PPF), are the only source of the diaphragm musculature, rather than multiple sources postulated in embryological texts. Also, the number of precursors populating this tissue primordium appears qualitatively to be no different in the nitrofen model of CDH than in control animals, so this aspect of diaphragm development does not appear to be the source of the pathogenesis in CDH. This work provides insight into the interrelationship between the phrenic nerve axons and the myoblasts and myotubes during diaphragm embryogenesis.

Chapter 4 addresses the question of the pathogenesis of congenital diaphragmatic hernia, namely Objective *ii*. In the field of CDH research, there are numerous theories about the origin of the defect. The main theories are that the defect arises from a problem with muscle formation, or it is the consequence of a primary lung defect. Studies described in this thesis have shown that the defect observed in the animal model occurs in the amuscular component of the diaphragm and independently of lung formation, discounting these prevalent theories. This supports the belief that the defect originates at an early stage of embryogenesis of the mesenchymal substratum of the PPF.

The third objective of the research was to investigate the etiology of CDH in an animal model, specifically by investigating the possibility that the defect was the result of a problem with the retinoid system of the embryo. The Vitamin A/retinoid system plays a critical role in embryonic development with retinoic acid functioning in regulation of the transcription of myriad genes. Chapter 5 discusses studies in which four compounds that produce diaphragmatic hernias in rodents were characterized and it was shown that all four share the common ability to inhibit retinoic acid synthesis. This supports the hypothesis that the retinoid system was involved.

Other diaphragm defects from genetically mutated mice were examined as a collaborative effort as detailed in Appendix 1. Two groups, one at Harvard University and the other at Washington University in St. Louis had generated mutant mice with diaphragmatic defects and contacted the Greer laboratory for its expertise in examining diaphragmatic tissue. Fetuses from these two lines were supplied, the defective diaphragms were isolated and immunohistochemical labeling with nerve- and muscle-specific antibodies was performed to gain an understanding of the pathogenesis of the defects. It was shown that a type of CDH occurs in *Slit3* null mutants that could be correlated to the septum transversum type of hernia seen in humans. From what is known about *Slit3*, this pathology in the nulls is unexpected and may provide insight into the genetic mechanisms regulating diaphragm formation. The *Fog2* mutant mice display a defect in which the muscle formation in the diaphragm and the innervation pattern is severely disturbed. This defect resembles the diaphragm eventration developmental defect in humans. The molecular mechanism controlling diaphragm development is not understood. Gene-knockout mouse models will start to provide insight in this regard.

## *1.2 DIAPHRAGM EMBRYOGENESIS*

Embryological and medical textbooks commonly present the diaphragm as formed by contributions from 4 distinct structures in the developing embryo (Carlson, 1994; Skandalakis, 1994; Larsen, 1997; Sadler, 2000). These are the septum transversum, the pleuroperitoneal folds, the dorsal esophageal mesentery and the lateral body wall mesoderm. According to these textbooks, the septum transversum, composed of mesodermal tissue, grows dorsally from the body wall caudal to the developing heart, separating the thoracic and abdominal cavities incompletely by the fourth week of human gestation. The septum transversum expands, fusing with the pleuroperitoneal folds and the dorsal mesentery of the esophagus. The dorsal mesentery forms the median portion of the diaphragm, and eventually the crural muscle develops from myoblasts that grow into this dorsal mesentery. The remaining muscular portion of the diaphragm (costal muscle) forms from muscular ingrowth from the body wall. Much of this is based upon interpretation of gross dissection of fetal tissue (Mall, 1910; Wells, 1954) and no rigorous systematic investigation of the origin of the muscle of the diaphragm appears to have been performed.

## 1.3 MYOGENESIS

The muscle of the diaphragm is derived from migratory precursors that originated at the level of the 3rd, 4th and 5th cervical somites (Brookes and Zietman, 1998). The somites are bilateral condensations of the paraxial mesoderm in the developing vertebrate embryo, and constitute the first visual signs of body segmentation (Keynes and Stern, 1988). The majority of skeletal muscle of the body is derived from the somites (Chevallier et al., 1977; reviewed by Hauschka, 1994). Somitogenesis begins on E8 in the mouse and proceeds rostro-caudally (Cossu et al., 1996). Cells in the ventral portion of the somite undergo an epithelial to mesenchymal transformation, forming the sclerotome (Denetclaw and Ordahl, 2000). Cells of the sclerotome will differentiate to form the connective tissue and cartilage of the vertebrae and ribs, and other structures in the body segment. The dorsal portion of the somite is composed of cells that remain epithelial. These dorsal cells form the dermomyotome (Borycki and Emerson, 2000) that will give rise to the axial and appendicular muscles of the body, as well as precursor cells for other tissues (Cossu et al., 1996). The medial part of the dermomyotome contains cells that migrate from the dorsomedial edge of the dermomyotome to form the subjacent myotome and differentiate to form the axial muscles (Denetclaw and Ordahl, 2000). Cells in the lateral portion of the dermomyotome migrate from the ventrolateral edge and migrate to populate the limb bud and form limb muscles (Christ et al., 1977) and other muscles in the embryo (Ordahl and Le Douarin, 1992). The number of precursor cells that migrate to form the limb and diaphragm is not exactly known, but each somite gives rise to only about 30 to 100 migratory muscle precursors (Megeney and Rudnicki, 1995).

These limb and diaphragm muscle precursors migrate to the level of the presumptive limb. Here a population of precursor cells continues migrating and invades the PPF, while the remainder enter and populate the developing limb bud. *Pax-3* is expressed in myogenic precursors that migrate to the limb buds from the dermomyotome (Goulding et al., 1991). This identification of *Pax-3* as a factor that would mark muscle precursors was confirmed by results from mutant mice (Splotch mice) having a *Pax-3* mutation and lacked limb muscles but had normal body wall and back muscles (Franz, 1993; Bober et al., 1994). *Pax-3* expression can be used to follow the muscle precursors during migration to the PPF. However, other factors are also required for this process. HGF/SF, a chemoattractant, and its receptor c-met are necessary for proper muscle precursor migration behaviour (Sonnenberg et al., 1993). The ligand and its receptor regulate growth, motility and morphogenesis (Sonnenberg et al., 1993). *HGF/SF* is expressed at high levels in the mesoderm of the limb bud, suggesting that it does in fact act as an attractant for the migratory cells. Cells in the lateral tip of the dermomyotome express *c-met* (Yang et al., 1996), coincidental with *Pax-3* expression. *Pax-3* modulates *c-met* expression in the lateral dermomyotome (Epstein et al., 1996). In splotch mutants, *c-met* expression is reduced in this region. In *c-met* nulls (-/-), the limb muscles and the diaphragms do not form (Bladt et al., 1995), whereas the axial muscles form normally which supplies evidence that c-met is also needed for migration of muscle precursors.

Only a small fraction of the migratory myogenic cells that exit the somites proceed from the entry point of the limb bud to the PPF. For these hypothesized progenitors to form the diaphragm there must be substantial proliferation to generate sufficient cells to form the entire diaphragmatic muscle. Muscle precursors express

*MyoD*, the first member of the *MyoD* helix-loop-helix family of transcription factors that was identified (Davis et al., 1987). *MyoD* is important in the development of appendicular muscles (Smith et al., 1994). Proliferation is marked by an increase in the number of *MyoD*-positive cells.

Differentiation begins as *Pax-3* expression becomes downregulated and replication ceases while members of the *MyoD* family, myoD, and later, *myogenin* (Wright et al., 1989), is concomitantly upregulated (Olson, 1992). This switch in expression of myogenic regulatory factors signals the muscle precursors to withdraw from the cell cycle and differentiate into myoblasts (Smith, 1994).

The muscle precursors populate the primordial diaphragm, the PPF that provides a foundation on which muscle can form. The exact make-up of this amuscular, early component of the diaphragm is not clear, but its hypothesized origin is the somatopleure. The somatopleure is composed of mesoderm and ectoderm and forms the body wall structures (i.e. somatic muscles, skeleton, and kidneys) and outer lining of the intraembryonic coelom, as well as the skin. The primary source of mesodermal cells that form the limb originates from the somatopleure at the level of the future limb (Christ et al., 1983; Geduspan and Solursh, 1992). Since the somatopleure contributes to the developing limb and constitutes the outer lining of the coelom/body wall (Christ et al., 1979, 1983), the PPF may also originate from this tissue, although evidence for this is not conclusive. Certainly, it is a likely source.

## *1.4 CONGENITAL DIAPHRAGMATIC HERNIA – EXPERIMENTAL AND CLINICAL ASPECTS*

### *1.4.1* ***Pathophysiology of CDH***

Congenital diaphragmatic hernia (CDH) has been a known condition for approximately 400 years, first described by Riverius (Bonetus, 1679) in a young man upon examination after death.

Holt (1701) gave the first account of a child born with a congenital diaphragmatic defect. Bochdalek (1848), an anatomist from Prague, described the posterolateral defect that most clinicians are familiar with and this condition still bears his name. This type of hernia is the most common type observed in infants. There are a variety of other diaphragmatic defects that occasionally occur congenitally, including the Morgagni hernia, named after an Italian anatomist, eventration of the diaphragm and the central or septum transversum hernia.

The Bochdalek or posterolateral diaphragmatic hernia is characterized by a hole or missing portion of the muscle in the posterolateral aspect (corner) of the diaphragm muscle. This is the most common congenital diaphragmatic hernia in humans and the defect is more commonly located in the left dorsal quadrant of the diaphragm (85-90%; Butler and Claireaux, 1962; Skandalakis, 1994) but does occur on the right and in rare instances, bilaterally. The defect can range from very small (1-2 inches) to comprising a considerable portion of a side of the diaphragm. The Morgagni hernia is a type of CDH consisting of a parasternal defect that occurs with a frequency of 1-2% in infants born with a diaphragmatic hernia (Carter, 1962; Stokes, 1991). Two other types of CDH are the central tendon (or septum transversum) hernia in which a hole exists in the nonmuscular tendinous medial region of the diaphragm and eventration of the diaphragm in which one leaf of the diaphragm is elevated abnormally by the pressure of the abdominal viscera. This occurs because the muscle sheet of the hemidiaphragm is thinner than normal or may have scattered muscle fibers or no muscle at all. There is an amuscular membrane where the normal leaf of the diaphragm should be (Skandalakis, 1994). The central tendon hernia is considered the least common and least understood CDH (Skandalakis et al., 1994). This defect is associated with a large opening (defect) in the central tendon, presumed to occur as a secondary rupture of the septum during development (Wesselhoeft and De Luca, 1984).

Congenital diaphragmatic hernia occurs in 1:3000 live births in humans (Langham et al., 1996), or with an estimated higher frequency in total conceptions because, due to associated defects, some affected fetuses do not reach term. The abnormalities are sufficiently large that, in 50% of cases, CDH can be detected during pregnancy by ultrasound or radiographic techniques between weeks 16 and 24; the usual finding with a Bochdalek hernia is that the stomach has entered the left side of the chest where the mediastinum has undergone a shift to the contralateral side (Bohn, 2002). Left-sided diaphragmatic defects are more easily diagnosed because hernias on the right side may not be detected if the stomach has not entered the thorax at the time of the examination (Bohn, 2002). The liver may seal or plug a hole in the right side, and since there is no actual herniation of the liver or other viscera into the thoracic cavity, defects on the right side may go undetected until the postnatal period. When the liver has entered the chest through a right-sided defect, indicating that the diaphragm defect is large, the prognosis is very poor (Metkus et al., 1996).

In some cases, CDH exists as an isolated defect. However, CDH can occur in multiple congenital anomaly (MCA) syndromes (Enns et al., 1998) possibly as the result of chromosomal abnormalities such as trisomies or specific chromosomal aberrations (~5%, Torfs et al., 1992; Steinhorn et al., 1994; ~18%, Bollmann et al., 1995). The diaphragmatic defect is merely one of many anomalies present in these cases.

Diaphragm formation is essentially complete by week 8 of gestation in humans (Skandalakis et al., 1994), when the diaphragm has completely formed a partition between the abdomen and pericardial cavity. In CDH, the diaphragm fails to form completely, leaving a hole that can vary widely in size between the thorax and abdomen. There is high mortality (40-50%; Harrison et al., 1994), although when patients with just an isolated CDH are considered, the survival rate is around 84% (Nobuhara et al., 1996). The severity of the associated pulmonary hypoplasia and pulmonary vascular maldevelopment is an important determinant of the outcome for the infants.

Among survivors, the morbidity can be severe, with feeding, growth and development problems seen in many infants. Other problems that afflict survivors of CDH can be numerous and varied in nature with some nonpulmonary problems often observed after the repair of the defect. For example, gastroesophageal reflux (GER) and oral aversion is seen in many survivors following surgical repair (Vanamo et al., 1996; Muratore et al., 2001). GER is most often associated with esophageal dilatation, which is most common in CDH infants that required extracorporeal membrane oxygenation (ECMO), a cardiopulmonary bypass technique that is used in some cases of severe pulmonary hypertension, or any mechanical ventilation. Any prolonged intubation may also retard development of the swallowing reflex, leading to oral aversion (Muratore and Wilson, 2000). Another nonpulmonary deficit that can affect CDH survivors is sensorineural hearing loss (SNHL). This is thought to arise from the use of pancurium bromide as a muscle relaxant for infants with CDH in conjunction with mechanical ventilation and which has been associated with hearing loss in CDH patients (Cheung et al., 1999). This drug and other muscle relaxants are believed to have ototoxic effects, with preterm infants being highly susceptible to these effects (R. Lemke, personal communication). The exact mechanism by which the damage to the auditory system results is not known. Another potential reason for the association of hearing deficits with CDH is that cochlear function is dependent on sufficient oxygenation and perfusion (Haupt et al., 1993) and infants with CDH can experience severe hypoxia due to pulmonary hypoplasia increasing the risk of damage to the auditory system. One study reported the rate of SNHL at nearly 60% of survivors of CDH (with and without ECMO therapy; Robertson et al., 1998). The study was a regional study, so the incidence will vary among different treatment centres, as SNHL is dependent on the treatment protocol that CDH patients undergo, in terms of drugs used and ventilation techniques applied.

Associated pulmonary problems are the most frequent and severe problems associated with CDH. These arise because the abdominal contents (intestine, stomach, liver) can enter the thoracic cavity through the opening in the diaphragm early in gestation, reducing the volume available for the lungs to develop and expand at birth. This restriction results in severe lung hypoplasia and immaturity. Surfactant deficiency results from the developmental delay of lung epithelium (Leinwand et al., 2002). Infants with CDH have lungs containing fewer pulmonary vascular branches as a result of delayed development. The pulmonary circulation develops in concert with the lung. In CDH, the severity of the lung hypoplasia correlates with a reduction in the cross-sectional area of the pulmonary vascular bed (Levin, 1978). Also, the thickness of the vascular walls is increased in CDH due to increased muscularity of the vessels (Levin, 1978; Geggel and Reid, 1984). The vessels necessarily are more muscular to conduct blood through the hypoplastic lungs, in part due to the paucity of the vascular bed. Medical problems stemming from these effects, such as persistent pulmonary hypertension of the newborn (PPHN) and surfactant deficiency, contribute most to the morbidity associated with CDH.

### *1.4.2 Treatment of CDH*

Treatment of CDH is not as straightforward as simply repair or closure of the hole in the diaphragm. Often, the defect in the diaphragm can be repaired using a flap of muscle from the body wall or other source, or with a synthetic patch but the problems associated with maldevelopment of the lungs and the displaced organs in the thorax remain and require treatment.

Many strategies have been attempted to alleviate associated pulmonary problems, mainly designed to accelerate the maturation of the lungs and improve their function because respiratory problems are the most significant problems faced by survivors of CDH. These include mechanical ventilation such as extracorporeal membrane oxygenation (Wilson et al., 1991) or high frequency oscillatory ventilation (HFOV), and pharmacological treatments such as inhaled nitric oxide (NO), which is a method mainly for treatment of the pulmonary hypertension seen in infants with CDH. NO is a nonselective vasodilator of the pulmonary circulation and does not affect systemic vascular resistance (Pepke-Zaba et al., 1991; Finer and Barrington, 1997; NINOS, 1997), so it is used clinically to relieve the hypertension without causing systemic hypotension in the infants.

Much has been anticipated from *in utero* interventions in the treatment of CDH, such as *in utero* repair of the defect or tracheal occlusion to assist in lung development and expansion. Repair of the hole in the diaphragm requires reduction of the abdominal contents back into the abdomen. The use of a plastic abdominal silo may be necessary to overcome the reduced capacity of the abdomen as well as increased intra-abdominal pressure. In cases where the fetal liver has entered the thoracic cavity through the defect, return of the liver can cause kinking of the umbilical vein, which can result in fetal demise (Harrison et al. 1993). Unfortunately, *in utero* repair can lead to premature labour, and when the fetus is already severely compromised, survival of such infants is very low.

Tracheal occlusion has produced some promising results in animal models. In this technique, surgery is done *in utero* and the trachea of the fetus is ligated using an internal plug or an external clip to prevent fluid from exiting the lungs (Harrison et al., 1996). The fetal lungs produce a fluid similar in composition to physiological saline via a chloride-dependent mechanism. In a normal fetus, the fluid produced in the lungs flows outward through the upper airway into the amniotic fluid, due to a positive pressure difference in the lungs relative to the surrounding amniotic fluid, and the elastic recoil of the fetal lungs (Hooper and Harding, 2001). This egress of the fetal lung fluid is counterbalanced to an extent by the action of fetal breathing movements (FBMs). Such FBMs are due to the contraction of the diaphragmatic, thoracic, laryngeal, oropharyngeal, and nasal muscle groups (Harding et al., 1993).

Liquid volume in the lungs has been shown to be dependent on contractions of the diaphragm (Miller et al., 1993). With the trachea closed, the fluid produced by the lungs accumulates, and as a result the lungs expand to accommodate the increased volume. The growth of the lung can also push the invading abdominal contents back through the hole into the abdomen. Even though lung morphology was essentially normal in animals treated with tracheal occlusion and release, the levels and composition of surfactant were abnormal. This technique has not been encouragingly successful in humans (Harrison et al., 1996) but has shown promise in animal models like the sheep. Consequently, the procedure is continually evolving as improvements in methodology occur, such as the video-fetoscopic (Fetendo) technique of tracheal occlusion (Harrison et al., 1998).

The rationale for performing an *in utero* intervention for treatment of CDH has historically been based upon the findings that suggested early diagnosis of CDH (between 18 and 25 weeks), liver herniation into the thorax, and a low lung-to-head ratio were strong predictors of a poor outcome (Harrison et al., 1998). The decision to perform *in utero* surgical techniques has to be weighed against the reported high survival rate (80%; Bohn, 2002) when postnatal therapies such as ECMO (Kim and Stolar, 2000) and other ventilation methods (i.e. HFOV) are used.

An alternative to tracheal occlusion is maternal administration of glucocorticoids such as betamethasone during gestation to induce lung maturation that has shown benefits in the rat model (Oue et al., 2000). Antenatal steroids are given to help the lungs in the fetus become mature. Drugs like dexamethasone improve maturation of the lungs but are not mitogenic and do not improve lung growth. The procedure of maternal administration of glucocorticoids is a standard procedure for mothers at risk for preterm delivery, but it is not commonly used in cases of CDH (B. Thébaud, R. Lemke, personal communications). However, there are several complications associated with dexamethasone including suppression of the maternal and fetal immune systems increasing susceptibility to infections (fungal or bacterial; Pera et al., 2002). It must also be realized that glucorticoids have been associated with neurological morbidity such as decreased brain weight and neurological deficits when administered postnatally or for extended or multiple courses of administration (Doyle et al., 2000).

### *1.4.3 Animal models of CDH*

Very little is known about the pathogenesis or etiology of CDH in humans. However, it is thought that a deficiency of Vitamin A may be a part of the etiology. In rats, Vitamin A deficiency (VAD) in pregnant females causes CDH in 25% of the fetuses (Anderson, 1941; Warkany et al., 1948). One study in humans also shows a possible correlation between Vitamin A status and CDH (Major et al., 1998).

There are several models for CDH including teratogen-induced diaphragmatic hernia in rodents and surgically-induced lamb and rabbit models. In the lamb model, a diaphragmatic defect is surgically produced on day 90 of gestation in the fetuses (term = 145 days), with resulting lung hypoplasia and immaturity (deLorimier et al., 1967; Kent et al., 1972). An attempt to simulate the lung compression resulting from visceral invasion of the thorax in a lamb model involved inflation of a balloon in the left chest during gestation (Haller et al., 1976). This model has disadvantages in that the animals are costly, the number of offspring per pregnancy is low and the gestation period is lengthy. A surgical model of CDH in the rabbit fetus was developed (Fauza et al., 1994) where a hernia was surgically induced on E24 (term = 31 days), and resulted in features such as pulmonary hypoplasia as seen in other models and the human condition. In the rabbit, there are large litters and a short gestation time, both of which provide advantages over the lamb model, but the small size of the rabbits and fetuses make creation and utilization of the model technically more demanding (Wilcox et al., 1996).

In rodents, there is a well-established chemically-induced model of CDH that is commonly used (Kluth et al., 1990; Wickman et al., 1993; Alles et al., 1995; Alfanso et al., 1996; Allan and Greer, 1997). This nitrofen (a herbicide) model arose over 30 years ago during toxicological tests (Ambrose et al., 1971). Nitrofen was relatively non-toxic to adult animals, but caused developmental abnormalities in the lungs, heart, skeletal and diaphragm tissue in fetuses when they were exposed prenatally (Costlow and Manson, 1981). When nitrofen was administered as a single dose to the dam between the eighth and twelfth day of rat gestation, malformation (herniation) of the diaphragm was the predominant effect observed. The resulting defects were similar to those seen clinically in human infants in terms of the range in size and the location of the defects (Ambrose et al., 1971; Costlow and Manson, 1981; Kluth et al., 1990; Kluth et al., 1996). The degree to which the abdominal viscera invade the thoracic cavity is a function of the size of the muscle defect as is seen in the human condition. The resulting diaphragm defects closely resemble the Bochdalek hernias observed in human infants. The incidence of hernias is about 25% in CD1 mice (Gray et al., 1983) given nitrofen on E8 and 45% (Costlow and Manson, 1981; Kluth et al., 1990; Allan and Greer, 1997; Francis et al., 1999) in rats if nitrofen is given on E8-10 of gestation. The defects occur on the left side of the diaphragm in the majority of instances, but some right-sided or bilateral defects are found with nitrofen administration on E8. Lung hypoplasia is also observed in all fetuses from a treated animal. Interestingly, if nitrofen is given on E12, the defects are seen exclusively on the right side of the diaphragm. This may be related to left-right asymmetry (axis patterning) in the embryo, a process that may be regulated in part by retinoic acid (Levin, 1998). A system of genes involved in patterning regulates the sidedness of the organs of the embryo. In quails raised on retinoic acid or retinoid deficient diets, the embryos display asymmetry defects including *situs inversus*. This is hypothesized to be due to the ability of RA to activate genes such as *sonic hedgehog* and *nodal* that are asymmetrically expressed during embryogenesis and that can regulate each other in the chick (Zile et al., 2000). Perhaps the preferential incidence of the defect to a specific side may be related to transient retinoic acid deficiency resulting from teratogen exposure at a specific developmental age.

The majority of research in the field of CDH where the nitrofen model is utilized focuses on problems in lung development. Nitrofen is thought to act directly on the lungs, interfering with their development as well as on diaphragm formation (Irish et al., 1998; Keijzer et al., 2000; Guilbert et al., 2000). Lung hypoplasia occurs in the nitrofen model even in the absence of a diaphragmatic defect, producing an efficient way of modelling lung maldevelopment. The hypoplasia and immaturity of the lungs in the nitrofen model are very similar to those seen in human infants with CDH (Tovar et al., 1997). Both the surgically induced and nitrofen-induced hernia models are being used to address a number of aspects of CDH-related problems, including lung maturation, hypoplasia and pulmonary hypertension. These studies are not easily performed in humans due to ethical considerations, and the animal models are much more tractable to research, experimentation and therapeutic trials.

Both in the surgical lamb model and in the rodent nitrofen model, the resultant lung maldevelopment has been used to study processes and factors involved in lung maturation (Hill et al., 1994; DiFiore et al., 1994; Benachi et al., 2002; Leinwand et al., 2002; Guarino et al., 2002). It is well established that lung development depends heavily upon physical processes such as lung expansion and/or fetal breathing movements (Harding and Hooper, 1996; Kitterman, 1996). In the models of CDH, lung volume is reduced and the lungs upon examination are structurally and biochemically immature. Distention of the lungs by fluid and fetal breathing movements induce cell proliferation and gene expression in the developing lung (Hooper et al, 1993; Harding et al., 1993). Lung stretching is also important in cell differentiation. As the lungs develop, alveolar epithelial cells are undifferentiated but, in response to stretch, differentiate into type II alveolar cells that are important in surfactant production. Some of these cells form type I alveolar cells that are important for gas exchange. Cell type can be determined from expression of specific markers for type II cells such as surfactant protein C (SP-C). SP-C mRNA expression was decreased in models of lung hypoplasia (Piedboeuf et al., 1999). Other surfactants such as SP-A and phospatidylcholine are reduced in these models due to decreased numbers of type II cells.

Data from animal models have also demonstrated that lung stretching and maturation affect gene expression of certain growth factors. Platelet-derived growth factor B (PDGF-B) expression and protein synthesis in the fetal lung are enhanced when the lungs are stretched (Liu et al., 1995). It was also found that insulin-like growth factor II (IGF-II) production is increased with lung expansion (Hooper et al., 1993). FGF-10 levels decreased 10-fold in the rodent model of lung hypoplasia, and addition of exogenous FGF-10 substantially improved lung growth (Acosta et al., 2001)

As important as the investigation of the mechanisms underlying lung immaturity is the utilization of the models in assessing therapeutic interventions for improving lung development. As discussed earlier, a technique such as tracheal occlusion can be used to prevent egress of lung fluid from the fetus, permitting expansion of the tissues with increased fluid volume. This mimics the effects of mechanical forces on the lungs during development. With the animal models, it is possible to measure the effects of this treatment on cell number and type, as well as gene expression, allowing an assessment of the potential benefits of this modality if transferred to the clinical setting. In the lamb model that tracheal occlusion does improve lung growth and alveolar expansion, but if the occlusion is not released prior to birth, it decreases the number of type II alveolar cells that are necessary for surfactant production in the lungs. If the occlusion was removed prior to delivery, the number of type II cells was increased (Bratu et al., 2001b). Tracheal occlusion has been tested in conjunction with maternal administration of steroids with the rationale that the occlusion will accelerate lung growth and the steroid therapy will enhance differentiation and maturation of the lung cells (Bratu et al., 2001a; Bratu et al., 2001b). Another study performed in a rabbit model with prenatal administration of surfactant (intraamniotic) showed that such treatment improved lung growth and distensibility (Tannuri et al., 1998). The improvement in lung growth was comparable to that seen with tracheal occlusion, but occlusion does not increase the lungs' distensibility because of decreased surfactant production with tracheal occlusion.

In addition to steroid, surfactant and glucocorticoid therapy, Vitamin A therapy has been studied in the rat model. Thébaud et al. (1999) studied the effects of treatment of nitrofen-exposed rats with Vitamin A to determine if this may could the lung hypoplasia associated with the nitrofen/CDH model. Thébaud et al. (1999) demonstrated that high doses of Vitamin A given maternally improved the growth of the lungs of nitrofen-exposed fetal rats during gestation and resulted in a modest decrease in the incidence of CDH.

Pulmonary hypertension seen in CDH has also been studied using the animal models. The walls of the pulmonary vasculature are often thickened and more muscularized in human infants with CDH than in those that are normal (Geggel and Reid, 1984). Proliferation of smooth muscle cells in small pulmonary arteries has been observed upon post-mortem examination of infants (Levin, 1978). Clinically, this hypertension has been refractory to some common vasodilators, and the models have permitted investigation of the mechanisms involved. Nitric oxide is a potent vasodilator of the pulmonary circulatory system. One hypothesis that has been considered is that in CDH there is a defect in endogenous NO production in the vascular bed, causing a high level of vasoconstriction. However, it was shown that, at least in the main branches of the pulmonary arteries, nitric oxide synthase was expressed at normal levels (Karamanoukian et al., 1995). Endothelins have also been implicated in the pathophysiology of CDH in humans and the rat and lamb models (Kobayashi and Puri, 1994; Kavanagh et al., 2000; Thébaud et al., 2000). The role of endothelin (ET-1) that has both vasoconstrictor and vasodilatory actions, depending upon which receptor is activated, has also been examined in the lamb model of CDH

(Thébaud et al., 2000; Kavanagh et al., 2001). The pulmonary vasodilatory response to nitric oxide was intact in the CDH model, but that the endothelin response was abnormal. Enhanced vasodilatation was observed when the $ET_A$ receptor was activated (Thébaud et al., 2000) which provides a rationale for the treatment of infants with CDH with $ET_A$ receptor agonists.

The possibility is intriguing that a common mechanism exists which results in lung hypoplasia and diaphragm herniation. If there is a common mechanism, it supports the hypothesis that the retinoid system is important in the causation of CDH, as lung development is very dependent on retinoic acid (Chytil, 1985; Stahlman et al., 1988), and retinoic acid is synthesized by the lungs of the rat (Napoli and Race, 1987). In the teratogen model, if the retinoid system was disturbed, as predicted, it could be anticipated that multiple systems would be affected. This is one significant limitation to the concordance between the model and the human situation. In the animal model the lung hypoplasia may not be a secondary consequence of CDH, but may be one of a number of primary targets of the teratogens, while in humans there appears to be a direct correlation between the severity of the hernia defect and the degree of lung maldevelopment with the diaphragm defect being the primary cause of the secondary lung abnormality.

The nitrofen model is a useful tool to study the muscle defect, however, due to its resemblance to the human condition. The sheep model is likely of greater utility in studying the lung hypoplasia associated with CDH, but the defect in the diaphragm is surgically induced rather than developmentally induced. The teratogen-induced CDH model is currently the only suitable model for studying the pathogenesis of the muscle defect in CDH. The muscle pathology is similar to the human case but again this defect is, in a sense, artificial itself. There is no evidence linking an herbicide such as nitrofen to CDH in human infants, based on epidemiological studies (Torfs et al., 1992).

## *1.5* ***RETINOID SIGNALLING***

### ***1.5.1 Retinoid uptake, transport, storage and conversion***

Vitamin A and its derivatives are critical dietary components throughout life, particularly during embryonic development. The importance of retinol in normal development has been known for over fifty years, when studies of the offspring of Vitamin A-deficient pregnant rats revealed the occurrence of a vast spectrum of congenital defects (Anderson, 1941; Warkany et al., 1948; Wilson et al., 1953). These early studies also suggested that some of these deleterious effects could be offset with supplementation of the absent nutrient. The time point in gestation when the Vitamin A was administered was critical in determining which congenital malformations were prevented. Excess Vitamin A can also cause a variety of birth defects (Lammer et al., 1985), while retinoic acid (RA) is more potent in causing these effects, suggesting that it is a more important biological retinoid.

However, the importance of retinoic acid, the active metabolite of retinol, in development has only been realized in the last twenty-five years. The teratogenic potential of RA (Lammer et al., 1985; Goulding and Pratt, 1986; Rosa, 1987) and the identification of the nuclear receptors for RA and their regulatory actions (Giguère et al., 1987; Petkovich et al, 1987) solidified the critical roles played by RA.

Retinoids in the diet are found either as provitamin A carotenoids (from plant sources) or preformed retinoids (retinyl esters in animal tissues). These compounds undergo conversions in the lumen of the intestine to Vitamin A, by oxidative cleavage to form retinal, which is then reduced to retinol by retinal reductase (Olson, 1989). Retinal and retinol are absorbed by enterocytes in the intestinal mucosa. Inside the mucosal cells, these retinoids are bound to cellular retinol binding protein II (CRBP-II). Retinal is readily converted to retinol by retinal reductase when bound to CRBP-II. The retinol absorbed or produced inside the cells is then converted to retinyl esters by enzymes such as lecithin:retinol acyl-transferase (LRAT) and packaged into chylomicrons for secretion into the lymphatic system (Ong et al., 1987). In the lymphatic circulation, the chylomicrons are broken down enzymatically into chylomicron remnants, which are delivered into the systemic circulation and are removed from the circulation primarily by the liver. Parenchymal cells in the liver take up the remnants. The retinyl esters are hydrolysed, forming retinol, which is then bound to retinol binding protein (RBP). The parenchymal cells are the major sites of synthesis of RBP in the liver (Yamada et al., 1987). The retinol complexes with RBP and is transferred to the stellate cells for storage where retinol is esterified again and stored until required (Ong et al., 1988). Under normal physiological conditions, about 75% of the retinoids in the liver are stored as retinyl ester in these stellate cells. When the organism requires retinol, retinyl ester is hydrolysed to retinol. This retinol binds to RBP from the circulation and the complex is then secreted into the plasma for delivery to extrahepatic tissues. Vitamin A-dependent tissues absorb the retinol-RBP. A receptor for RBP on the cell surface has been hypothesized (Heller, 1975). Retinol and RBP dissociate quickly in the plasma and the retinol can rapidly cross the hydrophobic cell membranes (Noy and Xu, 1990). Some of this retinol is stored in extrahepatic sites such as the lung, kidney, adipose tissue, and bone marrow (Blaner and Olson, 1994).

Once the retinol has entered the target tissue, it is once again bound to CRBP. This bound retinol is converted to retinal via oxidation by the action of retinol dehydrogenase in the cytoplasm. The enzymes of the ADH (alcohol dehydrogenase) family control this process (Molotkov et al., 2002). Retinal is released from CRBP and is used as a substrate for retinaldehyde dehydrogenase to form retinoic acid (Niederreither et al., 1999). There are three isoforms of this enzyme in mammals: RALDH1, 2, and 3. During embryogenesis, retinoic acid production is not global; rather it takes place in specific cells at particular developmental stages in the embryo. RALDH2 is an essential enzyme for retinoic acid production during development, as its absence results in no detection of RA anywhere but the eye and embryonic lethality occurs by E8.75 in null mice.

The RA produced by RALDH2 is bound to cellular retinoic acid binding proteins (CRABPs) and delivered directly to the nuclei of the cells or further metabolised into other retinoic acid derivatives, such as 9-cis-retinoic acid or retinoid glucuronides by the cytochrome P-450 system. These are the active metabolites of retinol and retinoic acid outside the visual system, where retinal is the most important retinoid.

### *1.5.2 Retinoid receptors*

The retinoids are essential for many life processes such as vision, reproduction, differentiation, metabolism and pattern formation during development. Retinol is converted to all trans-retinoic acid (atRA or RA). Retinal functions as a chromophore in the visual system, while retinoic acid exerts pleiotropic effects via regulation of gene transcription. Like steroid hormones, retinoids are targeted by receptors in their target tissues (Yamamoto, 1985). A superfamily of ligand-dependent transcription factors has been identified, including steroid/hormone receptors, thyroid hormones, Vitamin D, and retinoic acid receptors (RARs). These initially were termed orphan receptors meaning that the ligand was not known, but it was found that one of these was activated by retinoic acid (Petkovich et al., 1987; Giguère et al., 1987). There are three different RAR genes (α, β, and γ) each having several splicing forms derived from alternate splicing or promoter usage (Kastner et al.1990), indicating another level of complexity to the retinoid-signalling pathway. The different isoforms of RAR are activated by retinoic acid. In addition to the retinoic acid receptors, a family of receptors termed RXRs (Retinoid X Receptors) with high homology to the RARs has been identified (Mangelsdorf et al., 1995). RXRs exist in three forms: α, β, and γ. RA at pharmacological levels can activate these receptors, but the specific ligand is hypothesized to be 9-cis-RA, although this is somewhat in question (Werner and DeLuca, 2001). The RXRs are also necessary for transcriptional activation by RARs, TRs, VDRs and other members of the steroid/hormone receptor family. RAR binds to RXR, forming a heterodimer that binds to DNA to activate transcription. RXRs can homodimerize or heterodimerize with other members of the receptor superfamily to regulate gene expression.

The RAR genes are differentially expressed during embryogenesis (Dollé et al., 1989) with the different isoforms exhibiting a tissue-specific distribution during development. RAR-α is ubiquitously expressed in the embryo, with the transcript for the receptor being found by *in situ* hybridization in all tissues (Kato et al., 1992). The β and γ transcripts have a mainly non-overlapping distribution, with β found in the liver, lung, heart and kidney, while γ shows highest expression in the testis, skin and lung (Dollé et al., 1990). Retinoic acid levels in the embryo regulate RAR- β; RAR- β is down-regulated in VAD and is up-regulated by the addition of retinoic acid to a VAD-animal (Haq et al., 1991). The RXRs are differentially expressed in embryonic tissue as well. RXR-α and β are more widely expressed whereas γ is found in a more restricted distribution (Rowe, 1997).

Considerable redundancy has been found in attempts to elucidate the functions of the RAR receptor types. Knockout mice with one RAR subtype deleted displayed no overt phenotype (Lufkin et al., 1993), suggesting that compensation by another receptor subtype must occur. However, when all of the isoforms of a particular receptor are disrupted, a distinct phenotype is displayed (Lohnes et al., 1995) which may mean that for normal development, at least, one isoform of a receptor must be present in the tissues where the defects are observed. The lack of a phenotype in the single knockouts may mean that the mutants may be very subtly compromised. These single mutants did not show any fetal VAD-like malformations. Experiments with two RAR receptors mutated recapitulated all the congenital malformations characteristic of fetal Vitamin A deficiency (Lohnes et al., 1995), strongly indicating that retinoic acid and its receptors are important regulators of development. Several malformations not attributed to VAD in the early nutritional studies (Wilson et al., 1953) were found when mutant fetuses were examined, which may mean that Vitamin A deficiency leads to inactivation of all RARs causing embryonic death. Of interest to this work, only compound mutants lacking RAR α and β showed diaphragmatic hernia among the resulting defects.

### *1.5.3 Regulation of gene function by retinoids*

RAR and RXR are members of a receptor family which can regulate gene expression by binding as dimers to DNA sequences known as hormone response elements (HRE) in the promoter regions of target genes (those which are sensitive to a hormone or hormone-like molecule; see Figure 1.1). These specific sequences occur as tandem repeats that are separated by 1 to 5 nucleotides. Analyses based on sequence homologies to known HREs made possible the identification of the response elements that were activated by the RARs and RXRs. These sequences are termed retinoic acid response elements (RAREs) and retinoid X response elements (RXREs). RAREs are present in genes that are sensitive to retinoic acid, while RXREs respond to RXR homodimers. Since RXRs can heterodimerize with many different hormone and vitamin receptors, it is sometimes referred to as "the master regulator of hormonal signalling" (Mangelsdorf et al., 1994). These heterodimers bind to the partner receptor's specific response element (i.e. RARE, TRE, VDRE, etc). It is important to note that binding of the response elements does not exclusively activate gene transcription, but can also repress activation (Kliewer et al., 1992). This negative regulation is also possible with RXR:RAR heterodimers. These dimers in most instances activate transcription. The mechanism through which this repression occurs seems to involve RAR interference with positive acting transcription factors. Many of the RA-responsive genes encode proteins that are involved in transducing the retinoid signal from one step to another. This indicates that the signalling pathway is subject to autoregulation, meaning that positive or negative feedback mechanisms exist for regulation. The promoters for all three retinoic acid receptor genes contain RAREs, as do the genes encoding CRBP-1 and ADH3 (alcohol dehydrogenase 3).

The strong dependence of normal development on retinoid production and gene regulation suggests that the abnormal development of the diaphragm in the nitrofen model and potentially in human CDH could result from a "malfunction" of the retinoid system during gestation. This idea will be discussed later in the thesis with respect to some data presented and possible future work experiments that will investigate the role of retinoids in CDH.

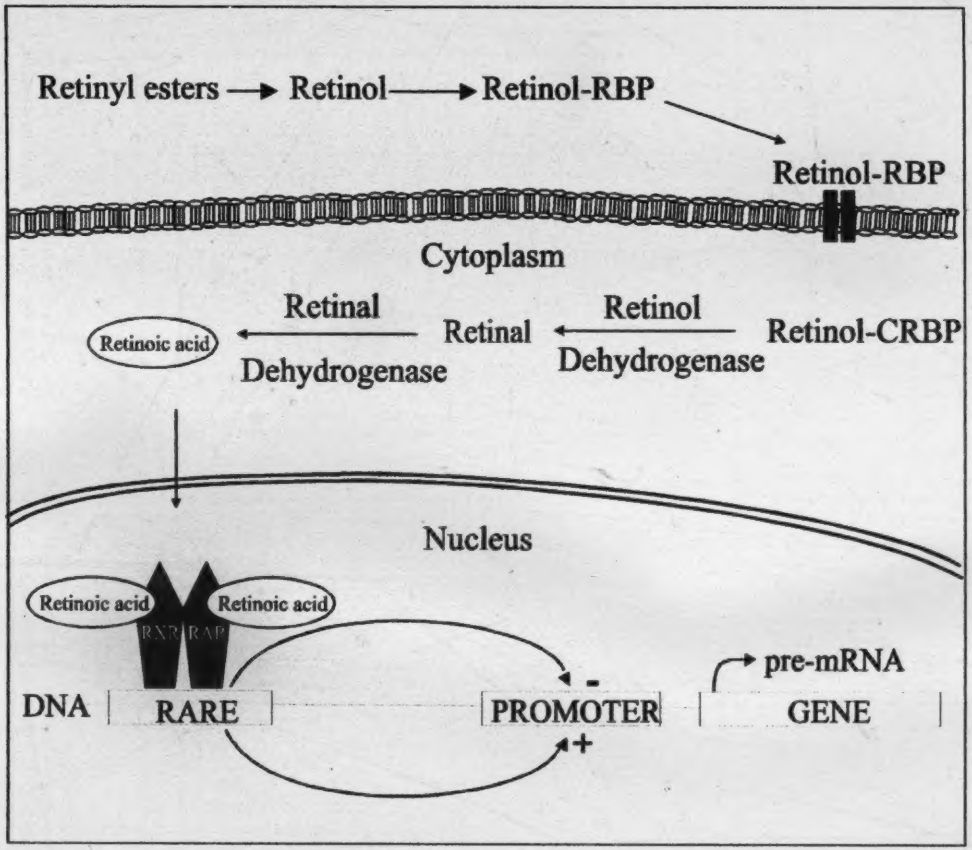
Retinyl esters
Retinol
Retinol-RBP
Retinol-RBP
Cytoplasm
Retinal Dehydrogenase
Retinal
Retinol Dehydrogenase
Retinol-CRBP
Retinoic acid
Nucleus
Retinoic acid
Retinoic acid
RXR
RAR
pre-mRNA
DNA
RARE
PROMOTER
GENE
-
+

**Figure 1.1 Simplified schematic of the retinoid-signalling pathway.**

Retinol bound to retinol binding protein (RBP) is transferred from the liver via blood to target cells. Retinol-RBP complex binds to cell surface receptors and is internalized. Within the cytoplasm retinol is bound to cellular retinol binding proteins (CRBP). Retinol is converted to retinal by retinol dehydrogenase followed by a further dehydrogenation to retinoic acid by retinal dehydrogenase. Retinoic acid (all-trans RA and 9-cis-RA) binds to receptors (RAR and RXR families) that dimerize and regulate gene expression by binding to short DNA sequences in the vicinity of target genes. (Figure from J.J. Greer)

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# CHAPTER 2. MATERIALS AND METHODS

### *2.1.1 Caesarean section (Rats and Mice)*

Staff of the Health Sciences Laboratory Animal Services (HSLAS) of the University of Alberta carried out all rodent breeding. Detection of sperm plugs in the breeding cages in the morning (designated E0) was used to determine the timing of the pregnancies. Timed-pregnant rat or mouse dams were anaesthetized with halothane (1-1.5%) using carbogen (95% $O_2$/5% $CO_2$) as the carrier gas, and maintained at 37° via radiant heat. Anaesthetized dams underwent Caesarean section and the fetal tissue was removed. Age was verified by comparison of the crown-rump length measurements with those published by Angulo y González (1932). Fetal rats were placed in 4% Paraformaldehyde and dissected to expose the diaphragm or interior of the body cavity (depending on the fetal age). To facilitate genotyping of mutant mouse tissue, fetal mice were initially put into 0.1 M phosphate-buffered saline (PBS), tail tissue was removed and frozen immediately, upon which the torsos were dissected and put into 4% paraformaldehyde.

### *2.1.2 Paraffin sectioning*

Fixed tissue was dehydrated through an ascending series of graded ethanol-water mixtures. Samples were incubated in xylene (x 2) before being transferred to plastic moulds containing liquid paraffin that were then incubated in an oven set to 55° C. After up to 3 days of incubation in liquid paraffin, the cups were removed from the oven and the tissue was immediately oriented as the wax cooled. Upon orientation of the tissue, the cups were cooled on ice to harden the wax. Embedded tissue was stored until sectioning. The paraffin blocks were removed from the moulds and trimmed down to facilitate mounting on the chucks of a 45 Rotary microtome

(Upshaw Manufacturing Co., Detroit, MI) with a standard knife holder and forward moving sample holder. Sectioning the blocks at 7 μm in thickness produced ribbons of sections that were floated in a water bath (40°C) to flatten and extend them. Ribbons of approximately 8 sections were mounted on Superfrost Plus slides (Fisher Scientific, Whitby, ON) and dried overnight in a slide dryer. Slides were stored in slide boxes for further processing.

### *2.1.3 Diaphragm isolation (E15-21 Rats; E14-E18 Mice)*

Fetuses were removed from the dam and fixed in 4% Paraformaldehyde. The torso above the level of the forelimb was cut away. Similarly, tissue including the hind limb and tail was removed to allow exposure of the diaphragm to the fixative. The tissue was fixed for 2 (room temperature) – 24 hours (4°C) prior to diaphragm isolation from the remainder of the torso. Diaphragms were washed thoroughly in PBS prior to immunohistochemical staining. Immunostaining was performed on free-floating diaphragms. The tissue was mounted on Superfrost Plus slides and photographed.

### *2.1.4 Immunohistochemical labeling*

Antibodies: Primary and secondary antibodies were diluted in PBS containing the appropriate normal serum (goat or rabbit) to a concentration of 1%. Dilutions, secondary antibodies and blocking serum used are described in the methods sections for the individual studies.

Labeling Protocols: Details of the immunolabeling protocol used for paraffin sections are found in the appropriate methods sections in the pertinent chapters. Free-floating diaphragms were labeled using the same solutions and washing regimens that were described for paraffin tissue sections. However, a routine antigen-unmasking step was performed for paraffin-embedded tissue. Dewaxed slides were immersed in 0.1 M citrate buffer and heated in a microwave for 5 minutes, cooled, and immunostaining was performed. Diaphragms were not subjected to this procedure. The immunohistochemical staining reactions were strong enough without treatment, and the procedure was damaging to the diaphragms. Omission of primary antibodies was used as a control for each immunolabeling experiment.

### *2.1.5 Microscopy*

Labeled tissue sections were photographed using a Leitz Diaplan microscope mounted with a CCD camera or a Nikon 990 Coolpix 3.37 megapixel digital camera. Images were captured by the CCD camera onto a computer with ImagePro software and subsequently transferred via Zip disk or network connection to a computer with Adobe Photoshop or CorelDraw software for processing while digital camera images were downloaded directly to a computer for image processing. Processed files were imported into CorelDraw for processing (contrast, brightness adjustment, cropping and labeling).

Mounted diaphragms were photgraphed as described for paraffin sections. Free-floating diaphragms were photographed while floating in the wells of 24-well culture plates or placed in 5cm culture dishes having a layer of 1% agar (in PBS). Pictures were taken with a camera mounted on a Leica Wild dissecting microscope (Leica, Germany). Processing of the images was carried out as described above.

Confocal microscope images of fluorescent-labeled tissue sections were taken on a Zeiss Axioplan microscope using a LSM 510 NLO laser interfaced with a computer running LSM 510 software.

### *2.1.6 Drug delivery*

Chemicals (teratogens, retinoids) were delivered to pregnant dams (rat or mouse) on Day 8 of gestation or on other multiple days as describe in the methods sections in the individual chapters. The appropriate masses of the chemicals were mixed with olive oil as a vehicle (1 ml for rats, 600μM for mice), vortexed and placed in a sonicating water bath for ~ 0.5 hours. Dams were anaesthetized briefly using Halothane as described earlier. The chemical solution was drawn up into a 3ml syringe to which a #5 French feeding tube was attached. The tube was passed through the animal's mouth, down its esophagus and past the cardiac sphincter into the stomach. The appropriate length of the feeding tube needed to reach the stomach was determined prior to gavaging by measuring the distance externally from the mouth of the animal to the midpoint of the abdomen. The syringe contents were expelled into the stomach by application of steady pressure to the plunger. Then the tube was removed and the animal was returned to the original cage and housed in the laboratory to await caesarean section.

### *2.1.7 C-met genotyping of adult and fetal mice*

Ear notches were collected by HSLAS from breeding age mice for genotyping to identify heterozygous animals for hetero-hetero crosses. Ear notch samples were digested in a buffer containing 5% Proteinase K to release the genomic DNA. Fetal tail samples were digested in the same fashion and genotyping on the samples was performed as described in the methods of Chapter 4.

## *2.2* ***REFERENCES***

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# CHAPTER 3. EMBRYOLOGICAL ORIGINS AND DEVELOPMENT OF THE RAT DIAPHRAGM

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Adapted from the Original Publication:

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R.P.B. contributed to the development of the hypotheses and experimental design, treated gavaged animals, did the embryo isolation, developed the immunohistochemical methods used, did much of the immunolabeling at early embryonic ages, diaphragm isolation at the earlier ages and performed the initial diaphragm labeling experiments.

W.Z. performed much of the embryo sectioning and immunofluorescence.

R.C. contributed to a large extent to the diaphragm isolation, immunohistochemical labeling and microscopy of the diaphragms.

D.W.A. provided input into the early design of the study and his work laid the basis for some of these experiments.

## *3.1* *INTRODUCTION*

Studies re-examining the embryological origins of the phrenic nerve and diaphragm have generated data to suggest that the classical view stated in most textbooks requires some fundamental revisions (Allan & Greer, 1997a, 1998). Specifically, the data do not support the model stating that the diaphragm musculature arises from multiple sources including the septum transversum, the esophageal mesentery and the musculature of the thoracic body wall. Rather, evidence indicates that the pleuroperitoneal fold (PPF) is the major, if not sole, contributor to diaphragm musculature. In this study, this hypothesis is tested more directly by examining the spatiotemporal distribution of muscle precursors and phrenic axons in the PPF and developing diaphragm of rats during the mid to late gestational periods. The nonmuscular component of the diaphragm is examined and the conclusion is that it may be a separate anatomical entity in itself.

An understanding of diaphragm development has clinical implications with regard to elucidating the pathogenesis and etiology of congenital diaphragmatic hernia (CDH), a developmental anomaly in which a portion of the diaphragm musculature fails to form. Rodent models of CDH were previously used to show that the initial defect could be traced back to a malformation of the PPF (reviewed in Greer et al., 2000b). Those studies are extended by examining the distribution of muscle precursors in tissue isolated from rats with diaphragmatic defects at various stages of development.

Central to meeting the objectives of this study was the ability to monitor the distribution of muscle precursors and axonal outgrowth at various stages of development. This was accomplished by immunolabeling for developmentally regulated molecules that are expressed in myogenic cells (Pax3, MyoD and myogenin; Bober et al., 1995; Muscat et al., 1995). The relationship between phrenic axon outgrowth and muscle precursor distribution was visualized via immunolabeling for neurofilament together with myogenic markers. To examine clearly the nonmuscular component of the diaphragm in the absence of the myogenic cells, mice mutant for c-met receptors, which bind HGF/SF, a chemoattractant necessary for guiding muscle precursors and axons to peripheral muscle (Bladt et al., 1995; Yang et al., 1996) were utilised.

## *3.2* ***MATERIALS AND METHODS***

### *3.2.1* ***Tissue preparation***

Fetal rats were removed via caesarean section from timed-pregnant rats anaesthetized with halothane (1.5% delivered in 95%$0_2$ and 5%$CO_2$) and maintained at ~37°C by radiant heat, as approved by the Animal Welfare Committee at the University of Alberta. The timing of pregnancies of dams was determined from the appearance of sperm plugs in the breeding cages; designated embryonic day (E)0. The ages of fetuses were confirmed by comparison of their crown-rump length measurements with those published by Angulo y González (1932). Embryos were decapitated and the thoracic and abdominal cavities exposed while immersed in 4% paraformaldehyde in a 0.2 M sodium phosphate buffer (pH 7.4). The tissue was then postfixed at 4°C for between 4-20 hours.

Whole embryo sections: After fixation, some fetuses were washed in phosphate buffered saline (PBS, pH=7.4) and placed in a 7.5% gelatin solution maintained at 30°C for 1 hour. They were then embedded in a 20% gelatin/glycerol solution that was hardened by cooling. A small block of the gelatin containing the fetus was excised and fixed overnight in 4% paraformaldehyde in a 0.2 M sodium phosphate buffer (pH 7.4). The block was appropriately oriented for transverse sectioning and vibratome sectioned at 50-70 μm intervals. Other embryos were embedded in paraffin and sectioned at 7 μm intervals using a microtome.

Diaphragm whole-mounts: Fetuses were fixed for 16-24 hours and diaphragms were isolated from the torso, washed in PBS, immunostained while free floating and mounted on Superfrost Plus® (Fisher Scientific, Can.). All photomicrographs of tissue were taken with a Nikon 990 digital camera and images were processed using Adobe Photoshop and CorelDraw.

### *3.2.2 Immunolabeling*

Antibodies: All primary antibodies used were diluted in PBS with 1% goat serum (Sigma, St. Louis, Mo.) and 0.4% Triton X-100. MyoD labeling was performed using a mouse anti-MyoD IgG MAb (clone 5.8A; 1:50-100 dilutions) obtained from DAKO Diagnostics Canada (Mississauga, Ontario, Canada). Myogenin was detected using a mouse anti-myogenin IgG MAb (DAKO and DSHB, University of Iowa, Iowa City, IA; clone F5D) at a dilution of 1:50-100. Pax3 was detected by using a polyclonal anti-goat Pax3/7 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:50. Myosin was detected using the MF20 IgG MAb developed by Dr. D.A. Fischman (Developmental Studies Hybridoma Bank, Iowa City) at a 1:5 dilution.

Neurofilament (165 kDa isoform) was detected using a mouse anti-neurofilament IgG MAb developed by Drs. T.M. Jessell and J. Dodd (Developmental Studies Hybridoma Bank) at a dilution of 1:100. Secondary antibodies, except rabbit anti-goat IgG, were diluted to 1:200 in PBS containing 1% goat serum. The secondary antibody used for MyoD, myogenin, and neurofilament was biotinylated goat anti-mouse IgG (Sigma: whole molecule). Biotinylated rabbit anti-goat IgG (Sigma) used for Pax3/7 was diluted 1:200 in PBS/0.4% Triton X-100 containing 1% rabbit serum (Sigma).

Immunohistochemistry: Tissues for all immunolabeling except Pax3/7 were immersed in PBS containing 0.4% Triton X-100 and 0.3% hydrogen peroxide for 20-45 minutes, followed by incubation in 1:20 goat serum (Sigma) in PBS for 1 hour. For anti-Pax3/7 immunolabeling the tissues were immersed in PBS containing 0.4% Triton X-100 and 0.3% hydrogen peroxide for 20-45 minutes, followed by 1:20 rabbit serum in PBS with 0.4% Triton X-100 for one hour. After 3 x 10 minute PBS washes, all tissues were incubated in diluted primary antibody over night at 4°C. After 3 x 30 minute PBS washes, tissues were incubated in the appropriate secondary antibody for 1-2 hours at room temperature, washed in PBS, then incubated in an avidin biotinylated-peroxidase solution (Vectastain ABC kit, PK-4000, Vector Lab.) for a further 1-2 hours. After PBS washes, antigen labeling was revealed by a DAB (3,3-diaminobenzidine tetrahydrochloride) product intensified with nickel ions [0.1M Tris buffered solution (pH 8) containing 0.04% DAB with 0.04% $H_2O_2$ and 0.6% nickel ammonium sulphate] for 5-15 minutes at room temperature. This produced an intense purple-black precipitate. After thorough washing, tissues were mounted and examined as above. Controls were provided by primary antibody omission or by use of an inappropriate secondary antibody.

Double fluorescent labeling: Paraffin sections were dewaxed in xylene for 4 min x 3, rehydrated in a series of ethanol solutions and washed in distilled water. Sections were then microwaved in 0.01 M sodium citrate buffer (pH 6) at 600W for 5 min and pre-treated with 1% hydrogen peroxide in 100% methanol for 30 min. Sections were treated with 0.1% bovine serum albumin in 0.4% Triton X-100/PBS for 30 min prior to incubation with mouse anti-MyoD monoclonal antibody (1:50, DAKO #M3512) and goat anti-Pax3/7 polyclonal antibody (1:50, Santa Cruz #sc-7748) overnight at room temperature. After incubation with primary antibodies and washing in PBS, the sections were incubated for 1.5 hour at room temperature with biotinylated rabbit anti-goat IgG (Chemicon) at a dilution of 1: 200. After washing in PBS, the sections were stained for 2 hours with a mixing solution that contained streptavidin Alexa Fluor488 (1:200, Molecular Probes #s-11223) and Alexa Fluor546 goat anti-mouse IgG (1:200, Molecular Probes #a-11030) to detect immunoreactivity of MyoD and Pax3/7, respectively. The stained sections were washed and cover slipped with Cytoseal.

Confocal laser scanning microscopy: Immunostained sections were scanned with a Zeiss Axioplan microscope (20X or 40X/1.3oil) using a LSM 510 NLO laser configured to a PC computer running LSM 510 software. For FITC fluorescence, excitation (Argon 25 mV) was at 488 nm and emissions were collected with a 505 nm long-pass emission filter. For rhodamine fluorescence, excitation (HeNe 1mV) was at 543 nm and emissions were collected using a 560 nm long-pass filter. Images were colour adjusted by using Adobe Photoshop 5.0 software.

### *3.2.3 Nitrofen model of CDH*

Nitrofen (100 mg), obtained from either the US Environmental Protection Agency or China National Chemical Jiangsu Company (Nanjing, China), was dissolved in olive oil (1 ml) and delivered via gavage feeding to pregnant dams temporarily (~10 min) anaesthetized with halothane (1.25%) on the evening of E8.

### *3.2.4 Amuscular diaphragm model*

C-met breeders were generously provided by Drs. Mark Tessier-Lavigne (UCSF) and C. Birchmeier (Berlin). For genotyping, embryos were removed from the yolk sac and embryonic membranes and tails removed and frozen for later DNA extraction. The torsos of the embryos were placed in 4% paraformaldehyde and stored at 4°C for anatomical and microscopic examination. Genomic DNA was prepared using a modified protocol using Proteinase K digestion, salt extraction and ethanol precipitation. Oligonucleotide primers (sequences and initial PCR protocol provided by Dr. Mark Tessier-Lavigne) WMet 8S (5'CTTTTTCAATAGGGCATTTTGGCTGTG3') and WMet 10 (5'GTACACTGG-CTTGTACAATGTACAGTTG 3') were used to amplify a 650bp fragment specific to the *c-met* wild-type allele. A 300bp fragment of the mutant *c-met* allele was generated using primers Neo1L (5'CCTGCGTGCAATCCATCTTGTTCAATG3') and WMet 5 (5'CACTGACCCAGAAGAGTGG3'). For the wild-type and mutant bands, the DNA underwent 43 cycles of amplification consisting of denaturing (94 °C, 40 seconds), annealing (65 °C, 30 seconds) and extension (72 °C, 15 seconds + 1 second/cycle). 10μl of the sample was run on a 1.2% agarose gel to analyze the results of the PCR reactions.

## 3.3 RESULTS

### 3.3.1 Muscle precursors and phrenic axons congregate within the PPF.

Figure 3.1A illustrates the anatomical location and shape of the PPF at E13.5. This is the stage at which the PPF has fully formed and is immediately prior to the start of myotube formation. The PPF is a wedge-shaped tissue that tapers medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum. Figure 3.1B demonstrates that the left and right PPF are the sites at which proliferating muscle precursors congregate, as demonstrated by immunolabeling for MyoD. Figure 3.1C shows a left PPF that has been immunolabeled for Pax3/7, which demarcates both neurons and muscle precursors (Mansouri & Gruss, 1988; Bober et al., 1994; Ziman et al., 2001). The point of nerve contact is consistently toward the medial aspect of the PPFs (also evident in Figure 3.1B).

Further illustrations of the spatial distribution of Pax3-expressing and MyoD-expressing muscle precursors are shown in Figure 3.2. At E13.5, both populations exist throughout the PPF with some cells expressing both transcription factors, presumably during the transition between proliferating to differentiating stages.

The PPF is shown at an earlier stage (E12.5) in Figure 3.3. Immunolabeling for Pax3/7 provides a visualization of muscle precursors in the region leading into the PPF. Pax3-labeled muscle precursors are also observed ventral to the PPF in the region of the brachial plexus. Note that no evidence was seen for muscle precursors populating the PPF from the septum transversum or esophageal mesentery as previously proposed.

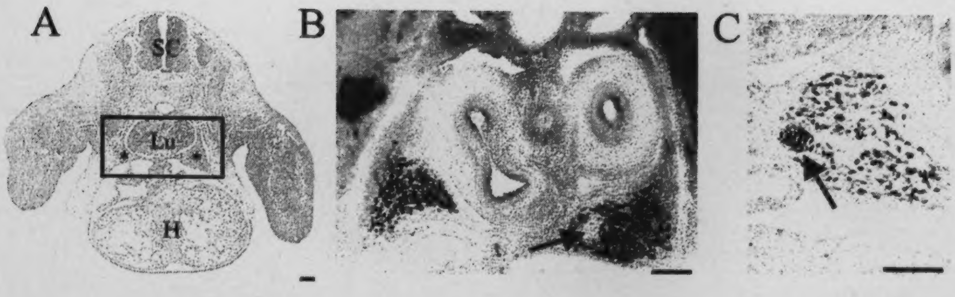

**Figure 3.1 The pleuroperitoneal fold (PPF) is the target tissue for phrenic axons and muscle precursors.**

A) Transverse section from an embryonic rat (E13.5) cut at the level of the mid-cervical spinal cord and stained with H&E. The area within the box contains the developing lung tissue and the bilateral pleuroperitoneal folds (*). B) Photomicrograph from an E13.5 rat embryo immunolabeled with an antibody to MyoD to delineate muscle precursors concentrated within the PPFs. Note that the area in the left PPF devoid of muscle precursors (arrow) is the site at which the phrenic nerve contacts. C) Left PPF immunolabeled for Pax3/7 that labels the nerve (arrow) and muscle precursors that have reached the PPF. Abbreviations: SC - spinal cord; Lu - lung; H – heart. Scale bars: = 100 μm

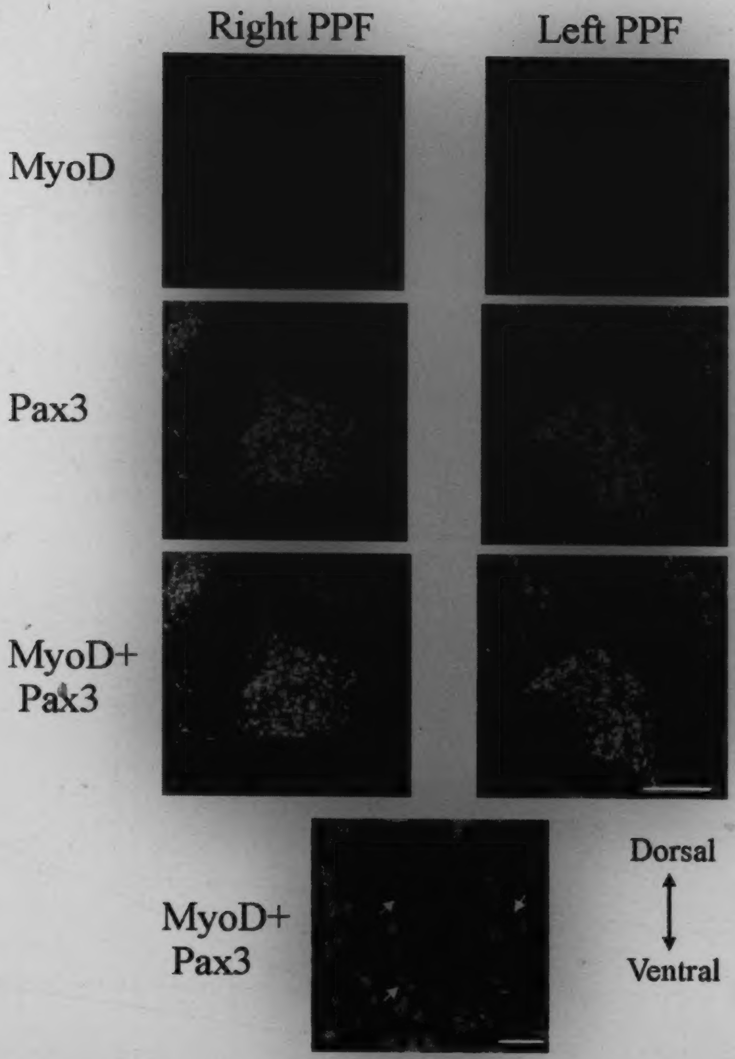
Right PPF
Left PPF
MyoD
Pax3
MyoD+
Pax3
MyoD+
Pax3
Dorsal
Ventral

**Figure 3.2 Pax3/7 and MyoD immunolabeling demonstrating muscle precursors within the PPF at E13.25.**

Confocal images show that Pax3 (green) and MyoD (red) labeled cells are present in the developing lateral wall musculature and bilateral PPFs. A significant number of muscle precursors express both Pax3 and MyoD (yellow) at this age. The arrows point to examples of cells that are expressing both proteins. Scale bars: = 100 μm; bottom panel = 25 μm

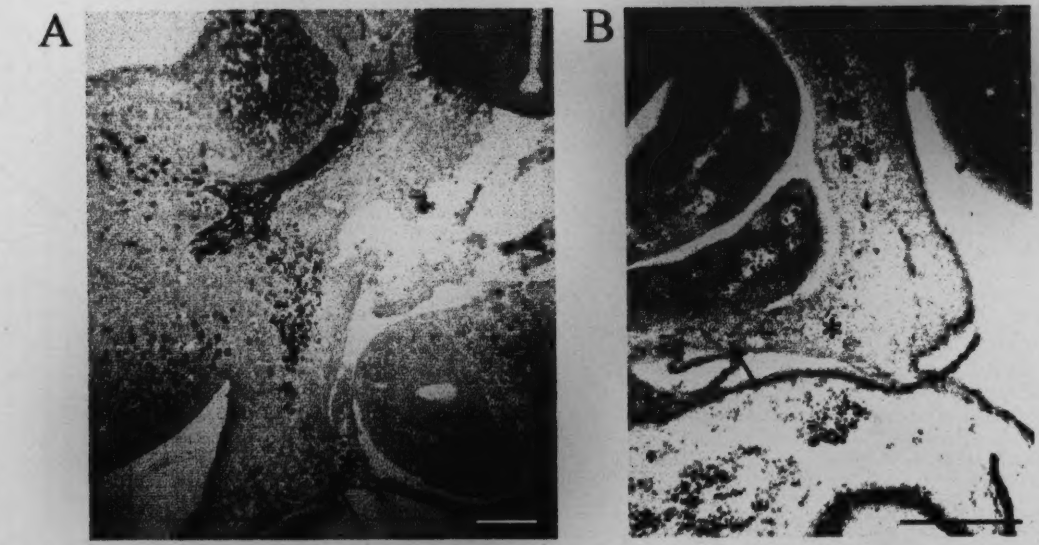

**Figure 3.3 Pax3/7 immunolabeling to demonstrate muscle precursors at E12.5 (arrows).**

A) Transverse section (7 µm) cut at the mid-cervical level of an E12.5 embryo. Pax3 labeled cells in the region leading into the right PPF (*). B) Pax3 labeled muscle precursors adjacent to, and within, a left PPF (*) at E12.5. Scale bars: A,B = 100 µm

### *3.3.2 Spatiotemporal pattern of muscle precursor migration/proliferation and intramuscular nerve branching*

From E14.5 through to E17, muscle precursors extend from the PPF into the dorsolateral costal, sternal costal and crural regions of the developing diaphragm. Figure 3.4A illustrates the spatial relationship between intramuscular phrenic nerve branches expressing neurofilament and muscle precursors expressing MyoD at E16. A higher magnified view in Figure 3.4B from another diaphragm illustrates that the muscle precursors are positioned ~150-200 μm ahead of the growing axons. In all diaphragms studied, the same relationship between the migrations of muscle precursors and axons were observed. Figure 3.4C shows the spatial relationship between muscle precursors and newly formed primary myotubes. In a subset of embryos, the thoracic musculature was left attached and immunolabeled the tissue for MF20 and MyoD (Figures 3.4C, D). This provided a clear view of muscle fibre and muscle precursor distribution in both muscle groups. No evidence for lateral wall muscle precursors contributing to developing myogenesis was seen, as previously proposed. Immunolabeling did not show any of the muscle precursors in the body wall flowing into the PPF.

Figure 3.5 shows the relationship between MyoD-expressing muscle precursors and phrenic intramuscular branches at E17. This is the stage at which the diaphragm is recruited for the generation of fetal breathing movements (Greer et al., 1992; Kobayashi et al., 2001). The phrenic intramuscular branches in each hemidiaphragm have progressed ventrally to a point where the tips have crossed sternally. This is not observed at later developmental ages, indicating that a retraction of nerve branches or differential growth of muscle to separate nerve endings occurs.

Muscle precursors can be seen at high density around the axon endings (Figure 3.5B). The muscle precursors, visualized by staining for MyoD, populate the entire region of future muscle fibre formation (Figure 3.5C).

Myogenin was also labeled. This is a transcription factor that is upregulated when muscle precursors have differentiated (Muscat et al., 1995). Figs 3.6A and B illustrate that myogenin-expressing cells are distributed adjacent to growing intramuscular nerve branches at E16. The extent to which myogenin-expressing cells are distributed around the axons is not as wide as observed for MyoD labeling (compare Figs. 3.6B and 3.4B). Myogenin-expressing cells are presumed to be those that have differentiated first and will fuse to form primary myotubes, with the as yet to be MyoD-expressing cells that were yet to be differentiated, positioned laterally. By E17, myogenin-expressing muscle precursors occupy the full extent of the muscularized diaphragm (Figure 3.6C). At E17, the myogenin labeling is particularly strong at the midpoint of the developing muscle where secondary myogenesis commences (Allan & Greer, 1998).

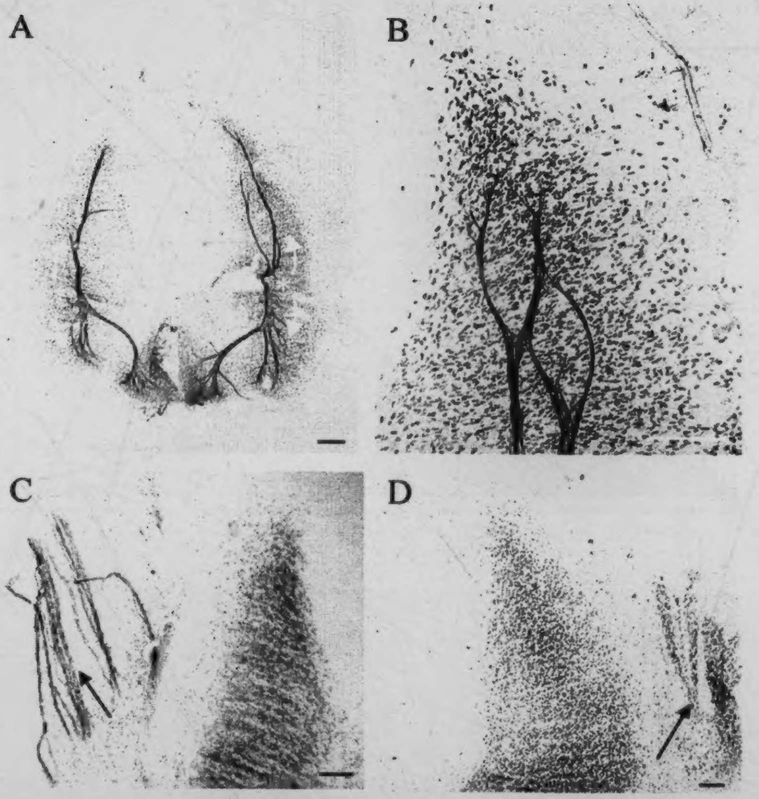

**Figure 3.4 Spatial relationship between phrenic intramuscular axonal outgrowth, location of muscle precursors and primary myotube formation.**

Whole-diaphragm isolated from an E16.5 embryo immunolabeled for MyoD and neurofilament to show the positioning of muscle precursors and phrenic axon terminals, respectively. Muscle precursors and phrenic axons radiate (arrows) from the location of the PPF (*). B) Higher magnified view showing muscle precursors localized in advance of, and around, extending phrenic axons. C) Left side of an E16 diaphragm showing the relative position of muscle precursors (MyoD-expressing individual cells) and myotubes (MF20-expressing striations). Note that the lateral body wall has been left attached with intercostal musculature (arrow). D) Right side of an E16 diaphragm with a portion of the body wall musculature attached labeled for MyoD. There is a well-defined separation between the muscle precursors in the developing diaphragm and body wall (intercostal muscles) without any clear evidence for muscle precursors streaming from the developing body wall muscle mass and contributing to the formation of the diaphragm musculature. Scale bars: A = 500 μm; B,C,D = 100 μm

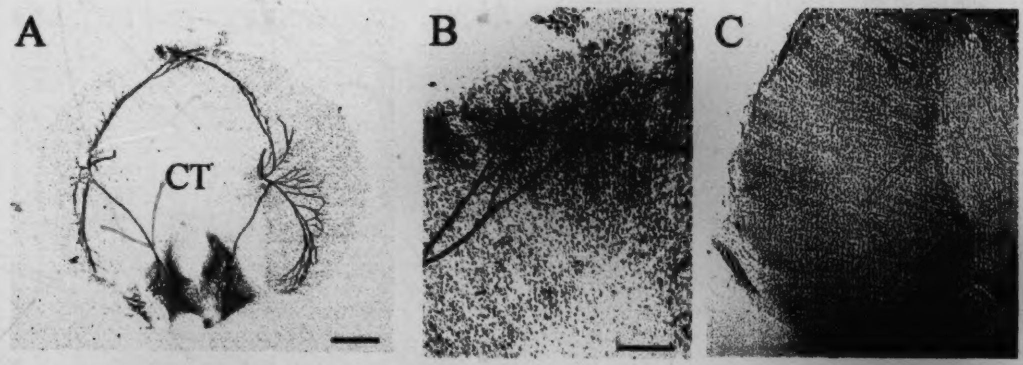

**Figure 3.5 Muscle precursors cover the full extent of the muscularized diaphragm by E17.**

A) Whole-diaphragm isolated from an E17 embryo immunolabeled for MyoD and neurofilament illustrating the respective position of muscle precursors and phrenic axon terminals. B) Close up of the most ventral aspect of the diaphragm showing the concentration of muscle precursors around the phrenic axon endings that have recently migrated to the regions. Note, that the right and left phrenic intramuscular branches are crossed over each other. This is not observed at later stages, suggesting that there may be some withdrawal of axonal endings. C) Left diaphragm immunolabeled for MyoD showing the full extent of muscle precursor distribution. Scale bars: A,C = 500 μm; B = 1100 μm. CT = central tendon

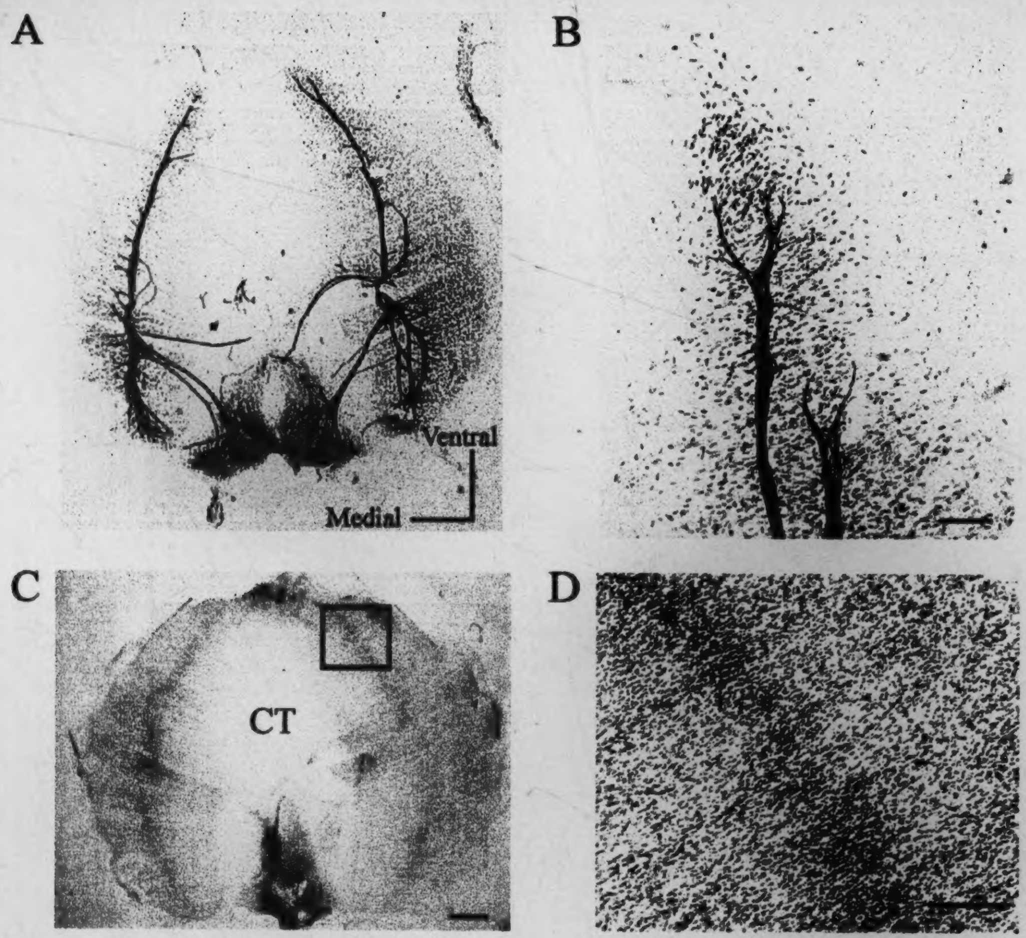

**Figure 3.6** **Distribution of differentiated muscle precursors expressing myogenin.**

A) Whole-diaphragm isolated from an E16.5 embryo immunolabeled for myogenin and neurofilament demonstrating the position of muscle precursors and phrenic axon terminals, respectively. B) Close up of the right side of another E16.5 diaphragm showing the concentration of myogenin-labeled cells around the phrenic axon endings. C) Whole-diaphragm isolated from an E17.5 embryo immunolabeled for myogenin showing the concentration of myogenin-labeled cells in the region where secondary myogenesis commences. D) Photomicrograph via a higher objective lens of an E17.5 diaphragm showing the dense myogenin labeling indicating the onset of secondary myogenesis. Scale bars: A,C = 500 μm; B,D = 100 μm. CT = central tendon

### *3.3.3 PPF and diaphragm formation in CDH*

In addition to the labeling of the PPF and developing diaphragms in control animals, corresponding experiments were performed on tissue isolated from the nitrofen model of CDH. In nitrofen-induced CDH, as reported previously (Allan & Greer, 1997b), there is a defect in the pleuroperitoneal fold (Figure 3.7A) with the liver protruding into the defective area (arrow; Figure 3.7B). MyoD labeling demonstrated that muscle precursors congregate within the remaining portion of PPF surrounding the defect. Immunolabeling for MyoD and neurofilament (Figure 3.7C) and myogenin (Figure 3.7D) of herniated diaphragms illustrates that the spatial distributions of intramuscular nerve branches and proliferating/differentiating muscle precursors is normal outside of the defective region. The area of the diaphragm that would normally arise from the defective dorsolateral PPF simply does not form.

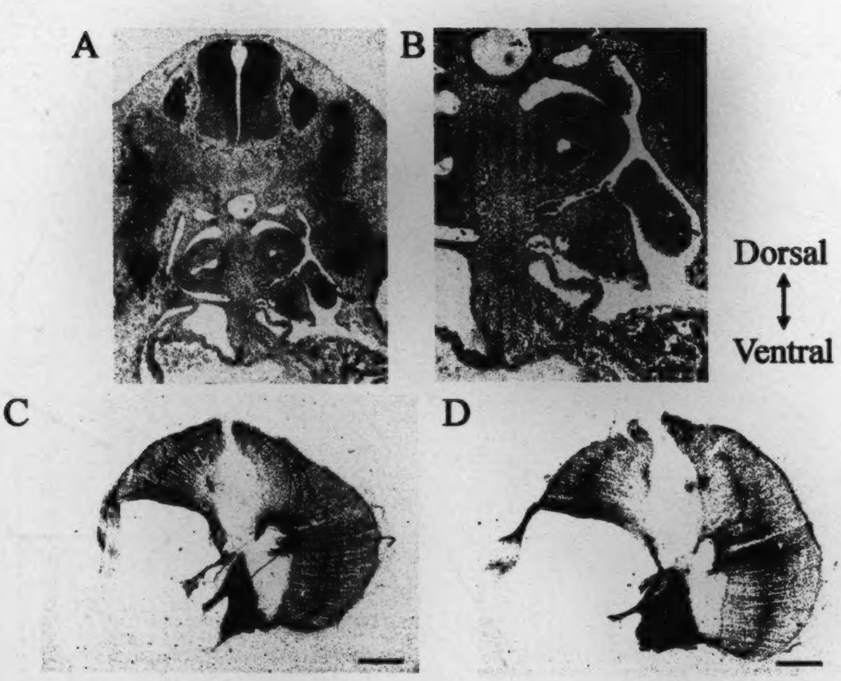

**Figure 3.7 Montage of diaphragmatic tissue at various stages of development from an animal model of congenital diaphragmatic hernia (CDH).**

Transverse section of an E13.5 rat exposed to nitrofen immunolabeled for Pax3/7. The dorsolateral region of the left PPF is missing and the underlying liver is protruding through (arrow). Muscle precursors occupy the remaining portion of the PPF. B) Higher magnified view of the left PPF shown in A. C) Diaphragm isolated from an E17.5 rat with a herniated diaphragm immunolabeled for MyoD and neurofilament. D) Herniated diaphragm isolated from an E17.5 rat and immunolabeled for myogenin. The distribution of muscle precursors and intramuscular nerve branches are normal outside of the defective region. Scale bars: A,B = 100 μm; C,D = 500 μm

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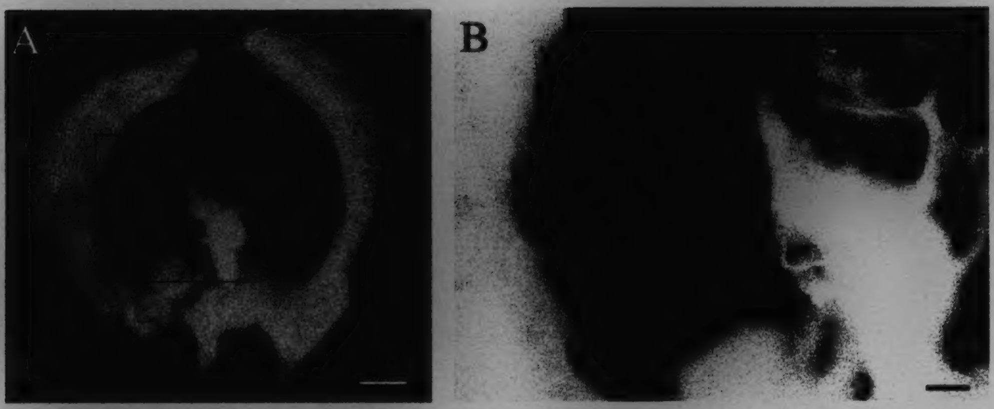

**Figure 3.8** **The amuscular component of the diaphragm forms independently from the myogenic component.**

A) Amuscular diaphragm isolated from a *c-met* null mutant mouse. Note that the connective tissue forms a continuous sheet that separates the thoracic and abdominal cavities. Openings for the vena cava, esophagus and aorta all form properly. B) Close-up of the region demarcated by the box in panel A illustrating the fibrous connective tissue sheet devoid of myotubes. Scale bars: A = 500 μm; B = 100 μm

## 3.4 DISCUSSION

Textbooks of embryology (Carlson, 1994; Larsen, 1997; Sadler, 2000) provide a standard set of drawings and text reflecting the traditional interpretation of phrenic nerve and diaphragm development based on anatomical dissections of embryonic tissue by Wells (1954). The advent of immunohistochemical markers for developmentally regulated molecules expressed by muscle precursors, growing axons and myotubes in conjunction with genetically engineered mutant mice has allowed for a more systematic examination of diaphragm embryogenesis. As discussed below, while some fundamental issues pertaining to diaphragm development remain unresolved, there are sufficient data to support a significant revision of narratives describing normal and pathological development of the diaphragm.

### *3.4.1 Sources of the diaphragmatic musculature*

Muscle precursors migrate to the brachial plexus region from cervical somites (Sze et al., 1995). It has not yet been demonstrated, but a subpopulation destined to form the diaphragm musculature may then migrate to the PPF. By approximately E12.5, muscle precursors have begun to reach the PPF where they begin to proliferate and differentiate. Post E13.5, there is a radiation of muscle precursors from the PPF toward the dorsolateral costal, sternal costal and crural regions of the developing diaphragm. The muscle precursors initially migrate to the central regions of these axes before spreading in mediolateral and dorsoventral directions in the costal and crural regions, respectively. By E17, muscle precursors have spread to all regions of the diaphragm that will be muscularized, leaving the area of the central tendon and a short tendinous zone separating the costal and crural regions amuscular. No evidence was found for contributions to the diaphragm musculature from the lateral body wall, septum transversum, or esophageal mesenchyme, as standard dogma would state.

Muscle precursors congregate and proliferate within the PPF prior to extending along the three major axes of muscle growth within the developing diaphragm. The identity of the guidance cues directing diaphragmatic muscle precursor migration and proliferation are unknown. Presumably, the cues are present on, and/or are released from, the underlying mesenchyme on which the diaphragm forms. HGF/SF is one potential candidate responsible for the guidance of muscle precursors to the PPF as null mutant mice the HGF/SF receptor (c-met; Bladt et al., 1995), fail to form muscularized PPFs and thus diaphragm musculature.

It has been postulated that the crural diaphragm is an axial muscle with separate embryological origins to the costal diaphragm (Langman, 1977; DeTroyer et al., 1981). This notion is based primarily on the fact that the crural region can be independently recruited during certain motor tasks (e.g. swallowing). The impression from the data is that muscle precursors located in the caudomedial aspect of the PPF contribute to the crural diaphragm musculature and there is no separation of embryological origins. Rather, as with many complex muscles, there is a functional compartmentalization of motor unit recruitment during specific motor tasks (reviewed in English et al., 1993). The data from the *c-met* null-mutants are particularly relevant to the question of the crural muscle being of a separate origin derived from axial precursors. In the null-mutants, muscles of axial origin form normally while those depending on muscle precursor migration do not (Bladt et al., 1995; Yang et al., 1996). There are no portions of the diaphragm that become muscularized in the null-mutants and thus the hypothesis that the crural region is of axial origins in not supported.

### *3.4.2 Relationship between the spatiotemporal distribution of muscle precursors, phrenic axons and primary myotubes*

Phrenic axons separate from brachial axons at the brachial plexus and migrate toward the PPF (Allan & Greer, 1997a, 1998). Diaphragmatic muscle precursors reach the PPF in advance of the growth cones of pioneer phrenic axons. Once phrenic axons reach the PPF, they consistently make contact with a specific locale within the medial region of the primordial diaphragmatic tissue. The molecular cues responsible for this stereotypical target recognition are currently unknown. Approximately 24 hours after contacting the PPF, the primary intramuscular branches of the phrenic nerve extend in the same three axes as the muscle precursors. The growth cones of the phrenic axons lag behind the advancing wave of muscle precursors and developing primary myotubes by ~ 200 μm. The muscle precursors may be providing guidance cues to the phrenic axons. Data from Harris (1981) and Lin et al. (2001) demonstrate that there are concentrated clusters of acetylcholine receptors located in the central region of developing myotubes in the absence of neural innervation. Thus, the postsynaptic specialization may be providing a target for the advancing intramuscular axons. Alternatively, there are molecular cues derived from the underlying mesenchymal substratum of the developing diaphragm that regulate both the migration and proliferation of muscle precursors and axonal outgrowth.

Past work has demonstrated that there is an elongation of primary myotubes bilaterally from the centre, where the nerve contacts, toward the periphery (Allan &

Greer, 1997a, 1998). Data from this study demonstrate that this can be accounted for by the spatiotemporal distribution of muscle precursors that proliferate and migrate along the three axes. In each of the axes, muscle precursors proliferate on either side of the centre and express MyoD. The precursors located most centrally differentiate first as shown by from myogenin expression. The differentiated muscle precursors fuse to form developing myotubes. By E17, when primary myotubes have formed throughout the full extent of the diaphragm, muscle precursors continue to proliferate/differentiate and are positioned adjacent to primary myotubes where they will contribute to the formation of secondary myotubes.

### *3.4.3 Amuscular component of the diaphragm*

Muscle precursors migrate to and proliferate/differentiate upon a mesenchymal substratum. This aspect of myogenesis in general is not understood. There is some evidence to suggest that the mesenchymal substratum is derived from the somatopleure (Tosney, 1988). It was somewhat unexpected to find that the mesenchymal substratum of the diaphragm formed a continuous sheet that completely separates the thoracic and abdominal cavity. Previous accounts of diaphragm embryology imply that the connective tissue portion forms the central tendon and fuses with the muscularized component to complete the diaphragm surface area. However, it appears that the musculature forms along and on top of the lateral edges of the substratum leaving the central portion amuscular (central tendon). The embryological origins of the nonmuscular component and how the septum transversum is incorporated are not understood and require further investigation.

Interestingly, a non-muscularized membrane separating the abdominal and thoracic cavities, referred to as the post pulmonary septum, are present in birds, crocodiles, turtles and some lizards (Kent, 1978). The purely speculative teleological argument that the nonmuscular component of the diaphragm and the septum of avians/reptiles share a common origin, but the mammalian diaphragm has adapted by the addition of a neuromuscular component, is proposed. Consider the following; muscle precursors destined for the mammalian diaphragm may migrate from cervical somites with muscle precursors destined for the forelimb. The two populations then diverge at the brachial plexus, presumably in response to distinct molecular cues. Similarly, phrenic and brachial axons take divergent paths at the brachial plexus. One could envision a muscularized and innervated diaphragm evolving from the divergence of muscle precursors and axons from the brachial population toward the mesenchyme of the PPF.

### *3.4.4 Distribution of diaphragm muscle precursors in an animal model of CDH.*

The nitrofen model has been utilized since the 1970's for studies of CDH (reviewed in Wilcox et al., 1996). The diaphragmatic defects in rodents exposed to nitrofen are similar to those observed in infants born with CDH. The pathogenesis of nitrofen-induced CDH can be traced back to the malformation of the PPF (Allan & Greer, 1997b; Greer et al., 2000a), rather than a failure of pleuroperitoneal canal closure, as often cited. The underlying mechanism by which nitrofen is acting is not completely understood, but there is evidence that it is interfering with the production of retinoic acid (Greer, et al., 2001, 2002), a key transcriptional regulator of embryonic development (reviewed in Ross et al., 2000). Data from this study track the distribution of muscle precursors in the PPF and developing diaphragm of normal and nitrofen-exposed embryos. Muscle precursors appear to congregate, proliferate and differentiate normally in animals with diaphragmatic defects. The striking difference between control and CDH tissue is the lack of regions of an underlying mesenchymal substratum in the PPF that subsequently contributes to the defective herniated region. Thus, these data are consistent with the hypothesis that the underlying amuscular component specific to the diaphragmatic anlagen is defective in CDH rather than a malregulation of myogenesis *per se.* If correct, then this explains why the diaphragm muscle is affected whereas other skeletal muscle is normal.

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# CHAPTER 4. DIAPHRAGM DEFECTS OCCUR IN A CONGENITAL DIAPHRAGMATIC HERNIA MODEL INDEPENDENTLY OF MYOGENESIS AND LUNG FORMATION

Adapted from the Original Publication:

Randal P. Babiuk and John J. Greer

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R.P.B. developed the hypotheses and experimental design, treated all mice, isolated the diaphragm tissue, and genotyped the *c-met* mouse tissue.

## *4.1* INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a serious developmental anomaly characterized by large regions of the diaphragm failing to form. Consequently the developing viscera invade the thoracic cavity and impair lung growth and development. As a result, newborns with CDH suffer from a combination of pulmonary hypoplasia, pulmonary hypertension, and surfactant deficiency (Harrison et al., 1994; Karamanoukian et al., 1995). CDH is associated with a 30-60% mortality rate and significant morbidity amongst survivors (Harrison et al., 1994; Nobuhura et al., 1996). Moreover, it is estimated that 1:2000 conceptions fail to reach term due to complications associated with CDH (Thorburn and Harding, 1994). Textbook explanations of the origins of CDH typically state that it is a defect in the fusion of the diaphragm muscle with the abdominal wall (i.e. the closure of the pleuroperitoneal canals) that results in the hernia. The pathogenesis of CDH is re-examined using a well-established animal model in which diaphragm defects characteristic of those found in infants with CDH are induced by administering specific teratogens midway through gestation (Costlow and Manson, 1983; Francis et al., 1999; Solomon et al., 2000; Sutherland et al., 1989; Tasaka et al., 1992; Wilcox et al., 1996). Data from those studies refute the hypothesis of a primary defect in the closure of the pleuroperitoneal canal (Allan and Greer, 1997). Rather, defects can be traced back to much earlier stages of development during the formation of the primordial diaphragm, the pleuroperitoneal fold (PPF). The PPF is a wedge-shaped tissue that tapers medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum. Myogenic cells and axons destined to form the neuromuscular component of the diaphragm migrate to the PPF and it is the expansion of these components that lead to the formation of the diaphragm (Babiuk et al., 2002). Three-dimensional reconstructions of the PPF have demonstrated that the malformed areas in the animal model of CDH are consistently located in the dorsolateral region (Greer et al., 2000a, Greer et al., 2000b). Correspondingly, the dorsolateral region of the diaphragm musculature is precisely the area affected in CDH. Thus, the embryogenesis of the PPF has become a focus for elucidating the pathogenesis of CDH.

Examinations of the muscle precursor migration to the PPF and subsequent proliferation and differentiation in the animal model of CDH did not reveal any obvious abnormalities (Babiuk et al., 2002). However, the underlying substratum of the PPF appeared abnormal. This has led to the current hypothesis stating that the amuscular mesenchymal component of the PPF, likely derived from the somatopleure (Tosney, 1988), is defective and does not provide a full foundation for the formation of diaphragmatic musculature. In this study, this hypothesis was tested using mice in which muscle precursors fail to migrate to peripheral muscle, including the diaphragm, due to homozygous mutation (-/-) of the c-met gene. The c-met protein, a receptor tyrosine kinase that is present on myogenic precursors, binds its ligand hepatocyte growth factor/scatter factor (HGF/SF), signalling migration of these cells (Bladt et al., 1995). While the diaphragmatic musculature fails to form in the null-mutants, the underlying connective tissue that comprises the amuscular substratum forms fully, thus, offering the opportunity to clearly visualize the formation of the amuscular component of the diaphragm in normal and teratogen-exposed animals.

A further hypothesis was tested that states that the lung hypoplasia associated with CDH is the primary defect that in turn causes the secondary defect in the developing diaphragm due to the loss of critical signals emanating from lung tissue (Cilley et al., 1997; Iritani, 1984). Central to that hypothesis is the notion that primordial diaphragm embryogenesis is regulated or influenced directly by the development of the adjacent lung tissue. Fgf10$^{-/-}$ null-mutant mice that do not develop lung tissue (Sekine et al., 1999) were used to address this issue. Specifically, whether or not lung tissue was necessary for 1) normal diaphragm formation and 2) the induction of diaphragmatic defects in an animal model of CDH was tested.

## *4.2* ***MATERIALS AND METHODS***

### *4.2.1 Mutant Mice*

Drs. M. Tessier-Lavigne (UCSF) and C. Birchmeier (Berlin) provided heterozygous breeding pairs of c-met mice. For genotyping, genomic DNA was isolated from ear-notch biopsies or fetal tail tissue according to standard protocols. Oligonucleotide primers WMet8s (5'CTTTTTCAATAGGGCATTTTGGCTGTG3') and WMet10 (5'GTACACTGGCTTGTACAATGTACAGTTG 3') were used to amplify a 650bp fragment specific to the c-met wild-type allele. A 300bp fragment of the mutant c-met allele was generated using primers Neo1L (5'CCTGCGTGCAATCCATCTTGTTCAATG3') and WMet5 (5'CACTGACCCAGAAGAGTGG3'). For the wild type and mutant bands, the DNA underwent 43 cycles of amplification consisting of denaturing (94 °C, 40 seconds), annealing (65 °C, 30 seconds) and extension (72 °C, 15 seconds + 1 second/cycle). 10

µl of the sample was run on a 1.2% agarose gel to analyze the results of the PCR reactions.

Dr. D. Ornitz (Washington University in St. Louis) provided breeding pairs of Fgf10$^{+/-}$ mice. Genotyping was performed by PCR using published primer sequences (26). A 383-bp fragment was generated from the wild-type Fgf10 locus by PCR using primers P1 (5'-CTTCCAGTATGTTCCTTCTGATGAGAC-3') and P2 (5'-GTACGGACAGTCTTCTTCTTGGTCCC-3'). The mutant Fgf10 locus was amplified using primers P3 (5'-ACGACGGGCGTTCCTTGCGCAGCTGTG-3') and P4 (5'-TCAGAAGAACCGTCAAGAAGGCGATA-3') to produce a 582-bp fragment. DNA was extracted following established methods from ear-notch biopsies and fetal tail samples and amplified for 30 cycles (15 seconds at 94 °C, 60 seconds at 60 °C and 90 seconds at 75 °C).

### *4.2.2 Drug Delivery*

Timed-pregnant animals were treated on E8 (embryonic day 8 of gestation) via gavage feed with CDH-inducing compounds dissolved in 600 µl of olive oil. Pregnant mice were briefly anaesthetized with Halothane. In the initial experiments, 25 mg/kg of nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether; China National Chemical Construction Jiangsu Company, Nanjing, China) was administered to dams on E8 based on past literature reporting a 25-30% occurrence of CHD in CD-1 mouse embryos (Cilley et al., 1997; Wickman et al., 1993). However, a very low incidence of hernias in embryos from c-met dams bred on a 12901A/C57BL6 background treated with 25mg/kg nitrofen (Table 4.1) was observed. The yield was too low to assay efficiently for hernias in the 25% of embryos homozygous for the c-met deletion. Thus, the protocol was modified, based on the fact that three other teratogens have been identified that induce defects similar to nitrofen (Greer et al., 2002). While a systematic study was not performed to study which of these compounds, or combination of, were most effective at inducing hernias, administration regimes that sufficed for testing the hypotheses in question were determined. The administration of a mixture of 14.5 mg of nitrofen and 14.5 mg of bisdiamine (Acros Organics, NJ) to induce hernias in c-met$^{+/-}$animals was used. A mixture of 14.5 mg nitrofen, 14.5 mg bisdiamine and 4.5 mg of SB-210661 (provided by Dr. H.M. Solomon, Glaxo SmithKline, King of Prussia, PA) was effective for inducing hernias in Fgf10$^{+/-}$ mice bred on a C57BL6/6X CBA background. Treated animals were returned to the original cage and housed in the laboratory.

### *4.2.3 Caesarean Section and Tissue Isolation*

On E14, mice were anaesthetized with Halothane and maintained at 37°C with radiant heat. The fetuses were delivered by caesarean section. Upon delivery of each fetus, a sample of tail tissue was collected and frozen for genotyping. Each fetus was photographed using a Nikon 990 digital camera mounted on a Leica research microscope. Fetuses were then decapitated and the thoracic and abdominal cavities opened to determine the presence of diaphragmatic hernia.

## *4.3 RESULTS*

Untreated *c-met*$^{-/-}$ mutant embryos appeared normal except for underdeveloped limb musculature due to impaired muscle precursor migration to peripheral muscles. (Figure 4.1A). The mutants also had amuscular "diaphragms" that were thin and lacked any visible striations indicative of muscle fibers (Figure 4.1B). Table 4.1 summarizes the incidence of hernias induced by the different treatment regimes used with the c-met mouse model. A combination of nitrofen and bisdiamine administered midway through gestation induced diaphragmatic hernias in ~40% of c-met mice, with the majority being left-sided. Similar to what has been reported in the nitrofen-mouse model (Cilley et al., 1997), treatment with teratogens produced a spectrum of other visible defects, including large facial clefts, exencephaly, polydactyly, and "loose skin" in some embryos. Eighteen fetuses were *c-met*$^{-/-}$ as determined by genotyping. Of these, 4 had clear defects in the amuscular diaphragmatic membrane. Figure 4.1C shows a photomicrograph of a defective diaphragm with the liver herniating into the thoracic cavity. The defective region was always located in the left dorsolateral region (Figure 4.1D). In size, the hernias covered from 40 to 70% of one side of the diaphragm. These defects were identical with respect to location, extent and visceral intrusion to those seen in nitrofen-treated rats and infants with CDH.

In Fgf10$^{-/-}$ mice, the trachea forms but the lungs do not develop (Sekine et al., 1999). Further, limb bud formation is initiated but outgrowth and muscularization of the limbs do not occur (Figure 4.2A). However, fully formed, well-muscularized diaphragms developed in all Fgf10$^{-/-}$ mice despite lung agenesis (Figure 4.2 B, C). Further, posterolateral diaphragmatic defects characteristic of CDH (Figs. 4.2 D, E) were present in animals treated with a combination of nitrofen, bisdiamine and SB-210661 (Table 4.1).

**Table 4.1 Incidence of hernias found in the mouse models with different drug regimens.**

| Mutant | Nitrofen<br># hernias | Nitrofen/ Bisdiamine<br># hernias | Nitrofen/Bisdiamine/ SB-210661<br># hernias |
|---|---|---|---|
| c-met | 2/50 embryos (from 8 dams)<br>2 left | 75/170 embryos<br>(from 20 dams)<br>59 left; 10 right; 6 bilateral | N/A |
| Fgf10 | 0/14 embryos (from 3 dams) | 0/12 embryos<br>(from 3 dams) | 6/26 embryos<br>(from 4 dams); 6 left |

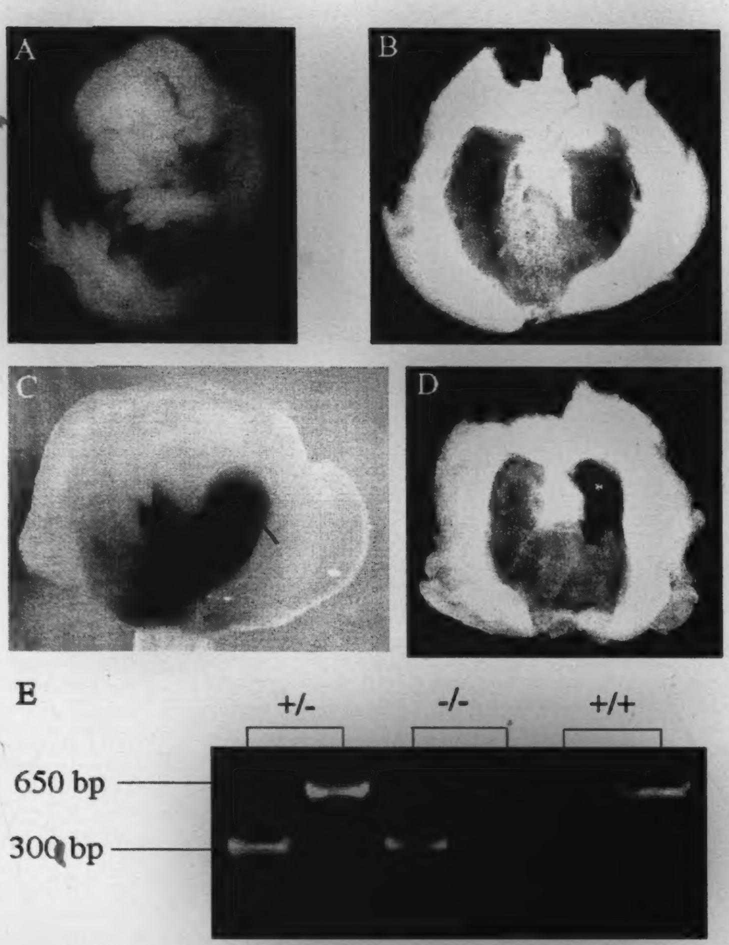

**Figure 4.1 Diaphragmatic hernias can be induced in amuscular diaphragms of *c-met*$^{-/-}$ mice**

A) Photo of an E18 *c-met*$^{-/-}$ mouse. The null-mutants have characteristic underdeveloped limb musculature due to abnormal muscle precursor migration to peripheral muscles. (B) Amuscular diaphragm with body wall attached from a *c-met*$^{-/-}$ mouse. The mesenchymal sheet is attached to the body wall laterally and the esophageal mesentery medially. (C) Photomicrograph of a diaphragm with a left-sided defect. The liver is protruding through the defect (arrow). Note that the remainder of the liver can be seen through transparent amuscular diaphragm. (D) Amuscular diaphragm with body wall attached from a *c-met*$^{-/-}$ mouse exposed to bisdiamine/nitrofen. There is a defect (*) in the left dorsolateral area of the diaphragm characteristic of CDH. (E) Genotyping of fetuses from heterozygous matings of c-met mice was performed as described in Materials and Methods. The three possible genotypes can be distinguished. +/+ , wild type; +/-, heterozygous; -/-, homozygous (null)

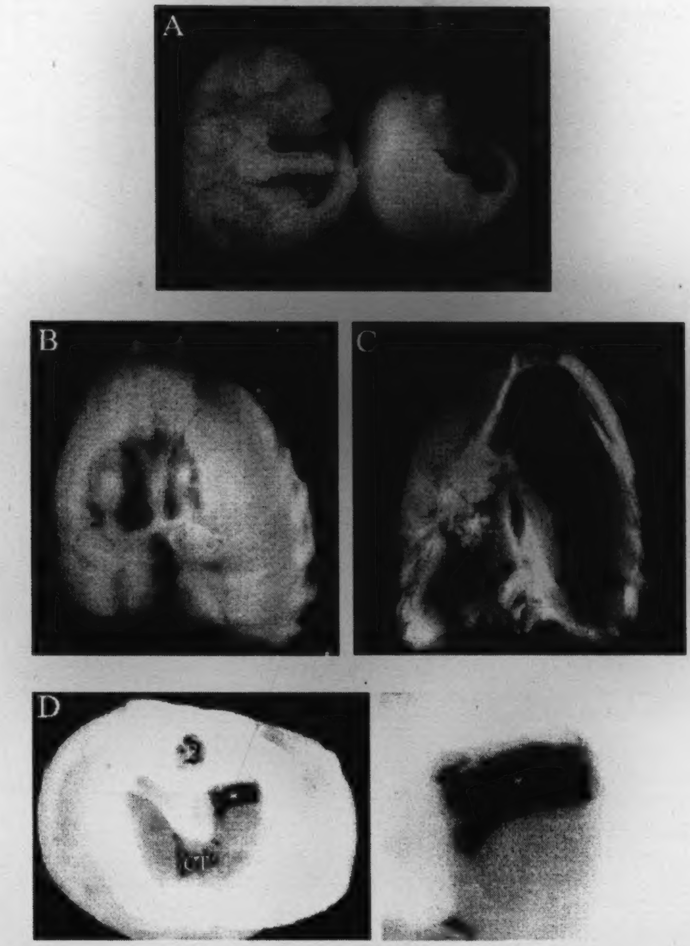

**Figure 4.2 In the absence of lung tissue, *Fgf10*$^{-/-}$ mice can develop diaphragmatic hernias.**

(A) Wild-type and *Fgf10*$^{-/-}$mice. The *Fgf10*$^{-/-}$mice have characteristic abnormal limb development. (B-C) Rostral and caudal views, respectively, of a diaphragm muscle isolated from an *Fgf10*$^{-/-}$ mouse. The diaphragm is well-developed without any noticeable abnormalities. (D) Diaphragm muscle with body wall attached from an *Fgf10*$^{-/-}$ mouse exposed to nitrofen, bisdiamine and SB-210661. There is a clear defect (*) in the left posterolateral area that is characteristic of CDH. (E) Close-up of the defective region. E = esophagus; CT = central tendon

## 4.4 DISCUSSION

The combination of the drug-induced models of CDH and mutant mice allowed us to test two fundamental hypotheses regarding the pathogenesis of this serious developmental anomaly.

### 4.4.1 Consideration of the animal model

The nitrofen-rodent model has been used widely to examine lung and muscle malformations associated with CDH. The use of this model arose from routine toxicological studies demonstrating that, while nitrofen was relatively non-toxic to adult animals, administration *in utero* resulted in ~50% of the fetuses developing diaphragm malformations that were remarkably similar to those seen in infants with CDH (Ambrose et al., 1971; Costlow and Manson, 1981; Kluth et al., 1990; Kluth et al., 1996). The similarities hold true with regard to the specific location and extent of diaphragmatic defects as well as the periodic occurrence of associated anomalies affecting cardiac, pulmonary and skeletal tissues. More recently, three additional compounds that cause diaphragmatic defects in rats have been characterized (Mey et al., personal communication). Biphenyl carboxylic acid (BPCA), bisdiamine [N,N'-octamethylenebis (dichloroacetamide)] and SB-210661, all induce diaphragmatic defects in the fetuses isolated from treated pregnant rats. BPCA is a breakdown product of a thromboxane-$A_2$ receptor antagonist, bisdiamine is a spermatogenesis inhibitor and SB-210661 is a benzofuranyl urea derivative developed for inhibiting 5-lipoxygenase. The timing of administration of all of the CDH-inducing teratogens is critical. Rodents are most susceptible between E8-11, a developmental window corresponding to gestational weeks 4-6 in humans. It was found that a combination of teratogens to be more effective in inducing hernias in the mouse strains used in this study. The precise mechanism of action of the CDH-inducing teratogens has not been elucidated, but recent data demonstrates that they all interfere with the retinoid signalling pathway by inhibiting retinaldehyde dehydrogenase (Greer et al., 2002).

### *4.4.2 Interpretation of data from experiments using c-met$^{-/-}$ mice*

The data derived from *c-met*$^{-/-}$ mice demonstrate that diaphragmatic defects can be produced independent of myogenic processes. This supports the hypothesis that the origin of the defect lies in the amuscular mesenchymal component of the primordial diaphragm, the PPF. These data provide a perspective on the mechanisms underlying CDH pathogenesis that is entirely novel from past theories. The focus now shifts from the muscularization of the diaphragm and closure of pleuroperitoneal canals to understanding the mesenchymal amuscular component of the diaphragm and how it is malformed in CDH. Further, the PPF mesenchymal substratum forms during the first 4 weeks of gestation and thus a reconsideration of the developmental stage at which the anomaly occurs is warranted.

### *4.4.3 Interpretation of data from experiments using Fgf10$^{-/-}$ mice*

Two primary conclusions arise from data derived from Fgf10$^{-/-}$ mice. First, the diaphragm can form normally in the absence of lung tissue and any putative associated growth related signals. Second, defects in the diaphragm in an animal model of CDH occur in the absence of lung tissue. The diaphragmatic defects associated with CDH are a primary defect and not a secondary result of lung malformation. The concept that the lung hypoplasia is in fact secondary to the diaphragmatic defect is supported by data from the surgically-induced sheep model of CDH that clearly demonstrate that a hole in the posterolateral diaphragm results in marked lung underdevelopment due to the invasion of abdominal contents and abnormal fetal breathing movements (Hill et al. 1994; Irish et al., 1994; Lipsett et al., 2000; O'Toole et al., 1997). However, there is convincing evidence demonstrating that the teratogens used in the rodent CDH model can directly interfere with lung development (Alfonso et al., 1996; Gilbert et al., 2000; Keijzer et al., 2000). Whether or not this is simply a reflection of the specific pathogenesis of teratogen-induced CDH remains unresolved. The possibility of there being a common mechanism underlying the pathogenesis of CDH that targets primordial diaphragm and lung development in parallel requires further investigation (Keijzer et al., 2000). Regardless, the pathogenesis of the lung associated with CDH, whether primary or secondary, must remain a focus of investigation if advances are to be made in the treatment of the disorder. These data demonstrate that an understanding of the pathogenesis of the diaphragm defect requires a focus elsewhere from the lung.

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# CHAPTER 5. RETINAL DEHYDROGENASE-2 IS INHIBITED BY COMPOUNDS THAT INDUCE CONGENITAL DIAPHRAGMATIC HERNIAS IN RODENTS

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*** Co-first authors that contributed equally to the study.**

**† Contribution to this work: development of the hypotheses and experimental design, characterization of the 4 teratogens (days of administration, dose levels)**

J.M. carried out the RALDH2 inhibition assays.

R.C assisted in teratogen administration and animal surgery.

W.Z performed the RALDH2 immunohistochemistry.

## *5.1* ***INTRODUCTION***

Congenital diaphragmatic hernia (CDH) is a serious developmental disorder occurring in approximately 1 in 3000 live births in which the diaphragm muscle fails to form completely, resulting in a hole in the diaphragm and incomplete separation of the thoracic and abdominal cavities. Lung hypoplasia and pulmonary hypertension are major pathological consequences that account for much of the morbidity and mortality (~50%) of this problem (Harrison et al., 1994; Karamanoukian et al., 1995).

An animal model of CDH was developed, resulting from toxicological studies which showed that nitrofen, a herbicide, while relatively harmless to adult rodents, caused developmental anomalies in the lungs, hearts, diaphragms and skeletal tissues of fetuses in pregnant rats (Ambrose et al., 1997; Costlow and Manson, 1981). Diaphragmatic defects resulting from a single 100 mg dose of nitrofen administered to pregnant rats on E8 of gestation are very similar to those documented in human CDH, with respect to the size and location of the defect and the accompanying intrusion of the abdominal viscera into the thoracic cavity. Further, the associated developmental defects observed with nitrofen-induced CDH such as skeletal and cardiac malformations are similar to those seen in a subpopulation of infants with CDH (Migliazza et al., 1999a; Migliazza et al., 1999b; Migliazza et al., 1999c).

Data derived from studies of the nitrofen model suggest that the pathogenesis of CDH is linked to a malformation of the primordial diaphragm, the pleuroperitoneal fold (reviewed in Greer et al., 2000). However, the etiology of CDH is largely unknown. Further, despite the fact that the nitrofen model of CDH has been used since the 1970's, a clear understanding of the mechanisms underlying the herbicide's teratogenicity is lacking. Given the striking similarities between the pathologies observed in CDH in the nitrofen-induced rat model and in infants with CDH, the possibility of there being a common underlying etiology certainly has to be considered. Therefore, the biochemical mechanisms underlying the actions of nitrofen were sought.

There are several pieces of data that provide a rationale for examining the role of the retinoic acid system in the etiology of CDH. Past studies examining the effects of Vitamin A deficient diets in rodents during pregnancy demonstrated that some of the offspring had diaphragmatic hernias (Wilson et al., 1953; Anderson, 1941). In 1994, Mendelsohn et al. published data showing that in a subset of double retinoic acid receptor subtype knockouts, fetuses had diaphragmatic hernias (Mendelsohn et al. 1994). Major et al. provided preliminary evidence supporting a role of Vitamin A as a factor in human CDH. In a small study of human mothers and infants born with or without CDH, it was reported that the retinol levels in the maternal and infant plasma were abnormal when CDH was present (Major et al., 1998). More recently, a direct interaction of nitrofen and the retinoid system arose from studies using transgenic mice with a LacZ reporter linked to a retinoid response element (RARE). The expression of the transgene was markedly reduced in response to nitrofen exposure (Greer et al., 2001).

In this study, the next step is taken by determining the specific stage in the retinoid cascade affected by nitrofen. Specifically, the hypothesis that nitrofen acts to inhibit retinal dehydrogenase-2 (RALDH2) and thus the formation of retinoic acid from retinaldehyde is tested. Further, three other compounds that induce diaphragmatic defects are characterized. Past reports have indicated that 4-biphenyl carboxylic acid (BPCA; Sutherland et al., 1989), bisdiamine [N, N'-octamethylenebis (dichloroacetamide); Taleporos et al., 1978; Tasaka et al., 1992] and SB-210661, (Solomon et al., 2000) induce diaphragmatic defects. BPCA is a breakdown product of a thromboxane-$A_2$ receptor antagonist, bisdiamine is spermatogenesis inhibitor and SB-210661 is a benzofuranyl urea derivative developed for inhibiting 5-lipoxygenase. These compounds induce diaphragmatic defects characteristic of CDH. Importantly, they all share, along with nitrofen, the same common mechanism of inhibiting RALDH2 in a dose-dependent manner.

## *5.2* *MATERIALS AND METHODS*

### *5.2.1 Animals*

Timed-pregnant Sprague-Dawley rats were used following procedures approved by the Animal Welfare Committee at the University of Alberta. The morning on which a sperm plug was observed in the breeding cage was designated as embryonic day (E) 0.

### *5.2.2 Chemicals*

SB-210661 was generously provided by Dr. H.M. Solomon (SmithKline Beecham Pharmaceuticals, Pennsylvania, USA). Bisdiamine [N,N'-octamethylenebis (dichloroacetamide)] was purchased from ACROS Organics (Fisher Scientific, Pennsylvania, PA) and 4-biphenyl carboxylic acid from Sigma (St. Louis, MO). Nitrofen was obtained from the US Environmental Protection Agency (Bethesda, MD) and China National Chemical Construction Jiangsu Company (Nanjing, China). The dosage and timing of administration for each chemical are listed in Table 1. Each compound was dissolved in 1 ml of olive oil using sonication. On the appropriate day(s) of gestation (Table 1), pregnant rats were anaesthetized with Halothane temporarily (10 minutes) and the drug solutions were delivered via gavage feed. Rats were returned to the original cage and housed in the laboratory for further dosing where specified.

### *5.2.3 Caesarean Section and Diaphragm Isolation*

On E18, rats were anaesthetized and caesarean sections performed to deliver the fetuses. The fetuses were euthanized, decapitated and placed into 4% paraformaldehyde for 1-2 days of fixation. Using a dissecting microscope, the diaphragms were then exposed and removed for subsequent assessment of defects. In some cases, immunolabeling for neural cell adhesion molecule (NCAM) was performed to delineate the orientation of myotubes and/or phrenic nerve intramuscular branches (Allan and Greer, 1997). Photographs of the diaphragms were taken with a Nikon 990 digital camera mounted on a Leica research microscope.

### *5.2.4 Retinal Dehydrogenase Assay*

To measure inhibitory effects on retinoic acid synthesis, the retinaldehyde dehydrogenase RALDH-2 isolated from an oligodendrocyte cell line (Richter-Landsberg et al., 1998; Mey and Hammelmann, 2000) was used in an assay. Trypsinized oligodendrocyte cells were collected on ice, spun down and triturated in an equal volume of 10 mM phosphate buffer pH 7.4 with 30 mM NaCl containing 1 mM PMSF, 1 μM leupeptin, 1 % aprotinin and 1 μM pepstatin as protease inhibitors. The homogenate was centrifuged for 15 min at 13,000 *g* to obtain a supernatant containing the cytoplasmic proteins. Protein concentrations in these extracts were determined with the BCA protein assay (Sigma). Isoelectric focusing (IEF) of native proteins was performed in an Isobox IEF apparatus (Hoefer Scientific/Pharmacia, Freiburg, Germany) with agarose gels using agarose-coated polyester film (GEL-Fix, Serva, Heidelberg, Germany), silanized glass plates and 1 mm thick plastic spacers. The gel solution contained 0.8 % agarose (Serva, 11402), 2 % sorbitol (Merck, Darmstadt, Germany) and 3 % ampholytes pI 4 - 7 (Serva, 42948). Electrode wicks were soaked in 0.5 M acetic acid and 0.5 M NaOH for anode and cathode, respectively. Samples were loaded with 10 μg protein per lane. Running conditions were: 10 min at 1 W; removal of the sample mask; 5 min, 5 W; 45 min, 15 W; - all at 1200 V maximum. Internal protein standards with pI 3.6, 4.6, 5.1, 6.6, 8.2, 8.6 and 8.8 (Sigma) marked pH positions in the gel. Lanes with pI markers were fixed and stained with Coomassie G250.

Following IEF, parallel lanes of the gel were cut into 16 or 24 consecutive slices 2.25 mm apart. These IEF fractions were distributed into the wells of microtiter plates, where proteins were eluted and assayed for RA synthesis from 50 nM all-*trans* retinaldehyde in the presence of 0.6 mg/ml dithiothreitol and 0.8 mg/ml NAD$^+$ (McCaffrey and Dräger, 1997). After 4 hrs of incubation at 37 °C in darkness, 50 μl/well of reaction products were removed and tested with RA sensitive reporter cells. The reporter cell line (Wagner et al., 1992) consists of F9 teratocarcinoma cells transfected with the β-galactosidase gene under control of the RA responsive element from the RA receptor β. The cells were grown in $CO_2$-buffered DMEM, supplemented with 10% FCS, penicillin/streptomycin (Sigma, P3539), and 0.8 g/l geneticin (Life Technologies).

For the RA assay, reporter cells were plated into polylysine coated 96-well microtiter plates, grown to confluence, and cultured for about 12 hrs with 150 µl cell culture medium plus 50 µl supernatant from the enzyme reaction. The RA-dependent induction of β-galactosidase was then visualized with the X-Gal staining procedure (Mey and Hammelmann, 2000). To measure the efficacy of enzyme inhibitors, IEF fractions were collected in medium that contained 0.1 µM, 1 µM, 10 µM or 100 µM of SB-210661, bisdiamine, nitrofen, or 4-biphenyl carboxylic acid (BPCA). After 15 min incubation at room temperature the $NAD^+$/RAL solution was added for the aldehyde dehydrogenase reaction as described above. To ascertain that enzyme inhibitors did not interfere with RA detection, the reporter cells were incubated with medium containing inhibitors plus 0.1 nM or 10 pM all-*trans* RA. Experimental procedures were identical to those when RA-production was determined. Aldehyde dehydrogenase activity was also measured in cytosolic extracts without prior IEF separation. In this procedure cytosolic extracts containing 10 µg protein were dissolved in 50 µl medium with enzyme inhibitors and then processed as described. Concentrations of 1 pM – 1 mM for nitrofen and BPCA, 1 nM to 1 mM for SB-210661 and 0.1 pM – 1 mM for bisdiamine were chosen. Three independent experiments were performed.

### *5.2.5 Immunohistochemistry*

Paraffin embedded embryos were sectioned transversely with a microtome at 7 μm intervals and mounted. Slides were dewaxed, rehydrated, then incubated with 0.3 % hydrogen peroxide in methanol for 30 min, and 0.4% Triton X-100/PBS for 1 hour. A blocking step was performed with 5% normal goat serum in 0.4% Triton X-100/PBS, followed by incubation with the primary antibody, rabbit anti-RALDH-2 (1:1500; P. McCaffery Massachusetts, USA) overnight at 4°C. The slides were incubated in a biotinylated goat anti-rabbit IgG (Vector) secondary antibody at a dilution of 1: 200 for 1 hour then washed in PBS. Slides were incubated with 1:100 avidin-biotinylated peroxidase complex (ABC kit PK4000, Vector). The antigen labeling was visualized by a DAB (3,3-diaminobenzidine tetrahydrochloride) product intensified by nickel (0.1 M Tris buffer containing 0.04% DAB with 0.04% hydrogen peroxide and 0.6% nickel ammonium sulphate). The slides were then counterstained with eosin and dehydrated in ethanol prior to being coverslipped. Photomicrographs were taken using a Nikon 990 digital camera mounted on a Zeiss research microscope.

## 5.3 RESULTS

### *5.3.1 Diaphragmatic Defects*

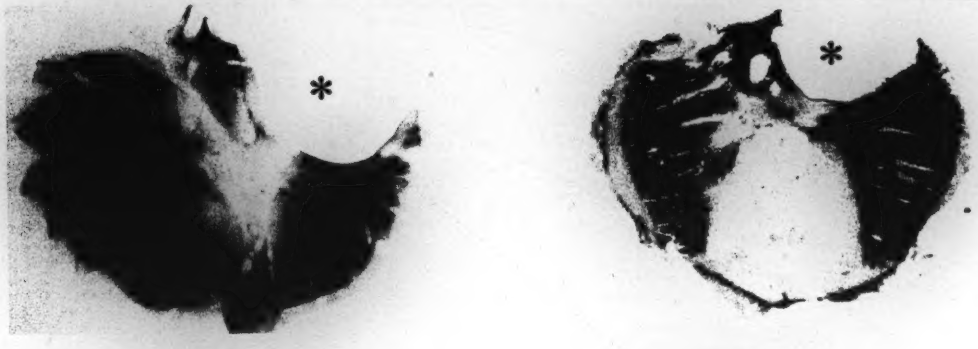
Fetuses removed from pregnant rats treated with nitrofen and 4-biphenyl carboxylic acid showed no outward abnormalities at the doses administered. A significant fraction (~50%) of the fetal rats from animals given high doses of SB-210661 (100 mg/kg/day, E7-14) or bisdiamine (100 mg/day on E10-11 or E11-12) displayed head abnormalities including "blunt" snouts and some skin defects ("loose" skin covering the trunk). Internal malformations were not systematically examined, although lung hypoplasia and retarded overall growth was evident from gross examination in the majority of fetuses exposed to the teratogens regardless of CDH status.

Figure 5.1 shows representative examples of diaphragm defects induced by the four compounds. The number of diaphragmatic defects produced by each of the compounds and data regarding the location of the diaphragmatic defects are contained in Table 5.1. As reported previously (Allan and Greer, 1997), a single dose of nitrofen administered on E8 results in ~50% of the embryos having hernias, with a predominance of left-sided defects. Past studies have also demonstrated that single doses of nitrofen administered beyond E11 induced solely right-sided defects. None of the other compounds induced diaphragmatic defects after a single administration on E8. Repeated doses were required.

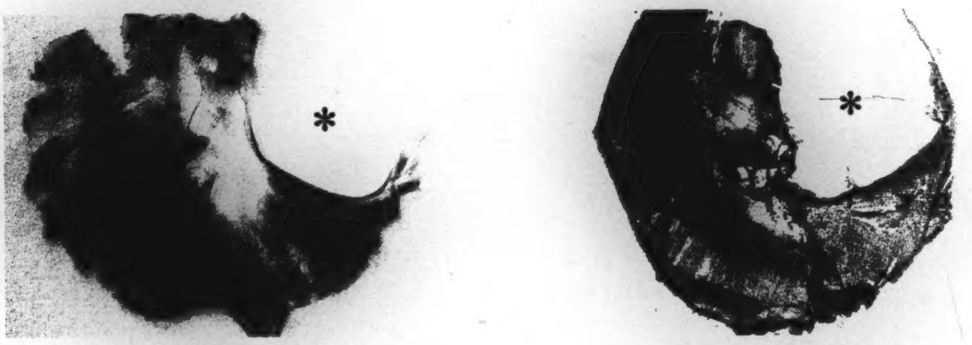
Administration of 4-biphenyl carboxylic acid at a dosage of 50 mg/kg/day from E7-E14 induced predominantly right-sided diaphragmatic defects. SB-210661, administered at a dose level of 100 mg/kg/day from E7-E14 caused 100% large bilateral defects; with ~80% of the diaphragm tissue missing in some cases. Doses of 50 and 75 mg/kg/day from E7-E14 induced defects of more moderate size in 74% and 85% of fetuses, respectively. Bisdiamine had a different dose-dependency. A 100 mg/day dose administered on E10 and E11 induced large bilateral defects. The same dosage administered on E11 and E12 induced only right-sided defects. Overall, the rank order of efficacy in producing diaphragmatic defects was bisdiamine > SB-210661 > nitrofen > BPCA.

**Table 5.1: Dosages and days of administration of teratogens and corresponding incidence and location of diaphragmatic defects.**

| Compound/Dose | | Days of Administration | Hernias/# of Fetuses | Location of Hernia (Left/Right/ Bilateral) |
|---|---|---|---|---|
| Nitrofen | 100 mg | E8 | 266/507 | 153L/86R/27B |
| BPCA | 100 mg | E8 | 0/10 | -- |
| | 50 mg/kg | E8-10 | 0/8 | -- |
| | 75 mg/kg | E8-10 | 0/12 | -- |
| | 50 mg/kg | E7-14 | 22/62 | 4L/16R/2B |
| SB-210661 | 100 mg | E8 | 0/13 | -- |
| | 50 mg/kg | E7-14 | 23/30 | 1L/14R/8B |
| | 75 mg/kg | E7-14 | 11/13 | 5L/6R/0B |
| | 100 mg/kg | E7-14 | 26/26 | 0L/0R/26B |
| Bisdiamine | 100 mg | E8 | 0/12 | -- |
| | 50 mg/kg | E8-9 | 2/10 | 1L/1R/0B |
| | 100 mg/kg | E8-9 | 1/14 | 1L/0R/0B |
| | 75 mg/kg | E10-11 | 7/18 | 3L/3R/1B |
| | 100 mg/kg | E10-11 | 6/17 | 0L/0R/6B |
| | 50 mg/kg | E11-12 | 16/16 | 0L/6R/10B |
| | 100 mg/kg | E11-12 | 2/17 | 0L/2R/0B |



SB-210661 Nitrofen



Bisdiamine 4-biphenyl carboxylic acid

**Figure 5.1 Photomicrographs of whole diaphragms labeled for neural cell adhesion molecule to demarcate myotubes.**

Representative examples of left-sided diaphragmatic defects (*) induced by each of the four compounds. The posterolateral portion of the diaphragm was affected in all cases.

### *5.3.2 Measurements of Retinal Dehydrogenase Inhibition*

Using the immortalized oligodendrocyte cell line OLN93 as a source of retinal dehydrogenase the inhibitory effect of nitrofen, bisdiamine, BPCA and SB-210661 on this enzyme were investigated. Since RA synthesis was assessed by means of beta-galactosidase expression in a bioassay the enzyme inhibitors were tested for their interference with RA-detection in this system. At a concentration of 0.1 μM none of the inhibitors had a significant effect on RA detection (Table 5.2), nor did 100 μM bisdiamine and nitrofen. SB-210661 reduced the reporter cell response at 100 μM to a small degree. At this concentration BPCA interfered with the zymography assay but not enough to render the RALDH inhibition data invalid (because BPCA inhibited RA synthesis completely and at much lower concentrations).

In zymography assays, IEF fractions were incubated with 0.1, 1, 10, 100 μM of nitrofen, BPCA, bisdiamine, SB-210661 or no enzyme inhibitor. All compounds reduced RA synthesis in a dose dependent fashion (Figure 5.2). Complete inhibition of the aldehyde dehydrogenase activity was found with all concentrations of bisdiamine, 10 μM nitrofen or higher, and 100 μM BPCA. Since only one peak of RA synthesizing activity was detected, cytosol homogenates were then used without prior separation by IEF to determine dose response functions for a broader range of concentrations (Figure 5.3). The $ED_{50}$ for suppression of RALDH activity were 10 – 100 μM for BPCA and SB-210661, approximately 10 μM for nitrofen, and below 1 pM for bisdiamine. In accordance with the zymography assays and the rank order of efficacy in producing diaphragmatic defects, bisdiamine, which suppressed RALDH activity at all tested concentrations, was the most potent inhibitor, followed by nitrofen, BPCA and SB-210661.

**Table 5.2** **Interference of enzyme inhibitor with retinal dehydrogenase detection assay. Complete inhibition of RA detection = 100, no inhibition = 0 (mean ± SD).**

| [Inhibitor] | Bisdiamine | Nitrofen | SB-210661 | BPCA |
|---|---|---|---|---|
| 0.1 μM | 0 ± 0 | 0 ± 0 | 0.3 ± 0.5 | 9.8 ± 24.1 |
| 100 μM | 7.5 ± 10.6 | 0 ± 0 | 17.5 ± 17.7 | 48.0 ± 7.4 |

### *5.3.3 RALDH2 Expression*

Immunolabeling for RALDH2 was performed to demonstrate its expression in the developing diaphragm, the pleuroperitoneal fold (PPF). As shown in Figure 5.4 there is intense labeling within the PPF at E13, the age when the structure is well defined. As reported previously (Malpel et al., 2000) there is also RALDH2 expression in the developing lung.

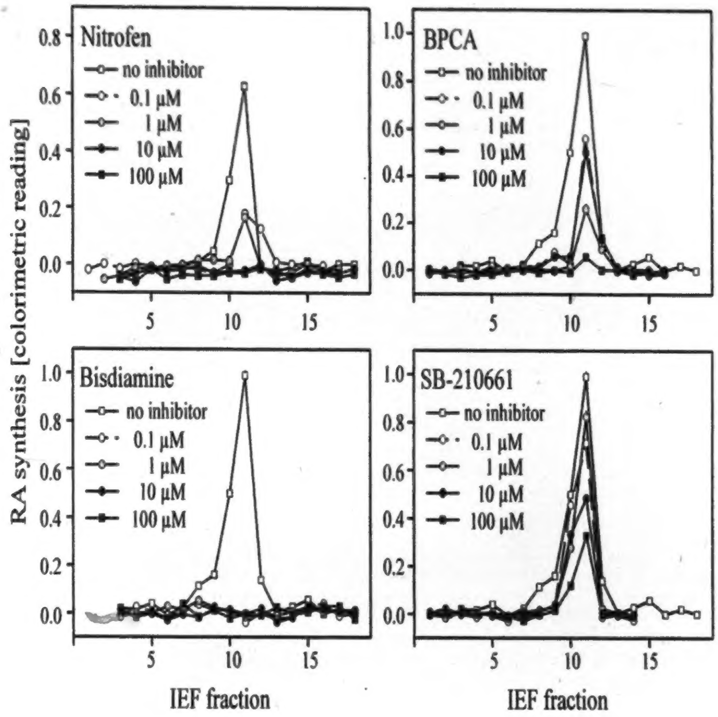


**Figure 5.2** **IEF fractions of cytosolic extracts from OLN93 cells exhibit RALDH activity.**

One peak of enzyme activity was detected in fractions with pI 5.0 – 5.6. Protein fractions were incubated with 0.1μM (-○-), 1μM (-□-), 10μM (-●-) or 100μM (-■-) of each of the 4 compounds or no enzyme inhibitor (-□-).

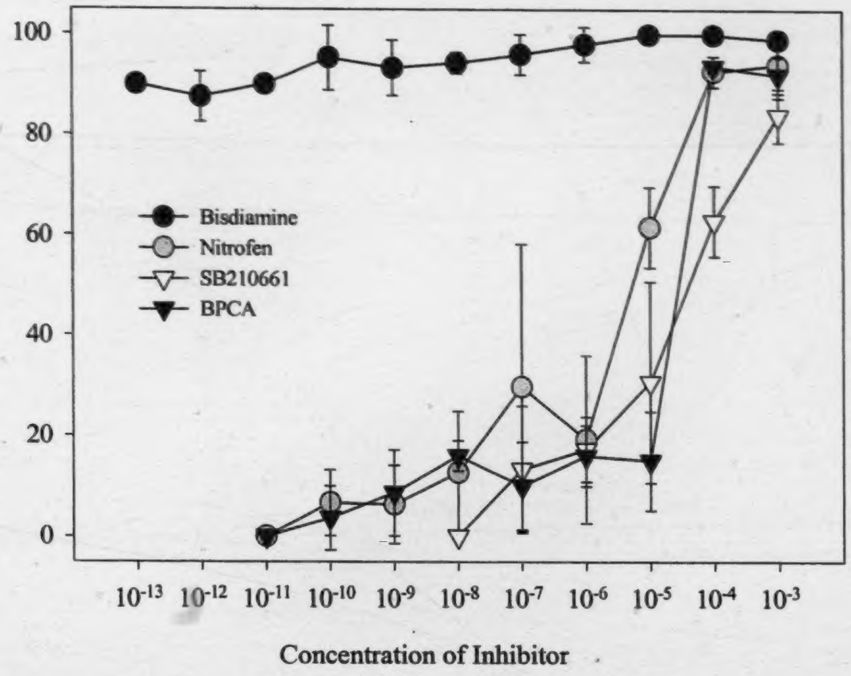


**Figure 5.3 Dose-dependent effects of enzyme inhibitors on RALDH2 activity.**

Relative inhibition on RA synthesis is plotted against concentration for each of the four compounds. Error bars indicate standard deviation of three independent experiments.

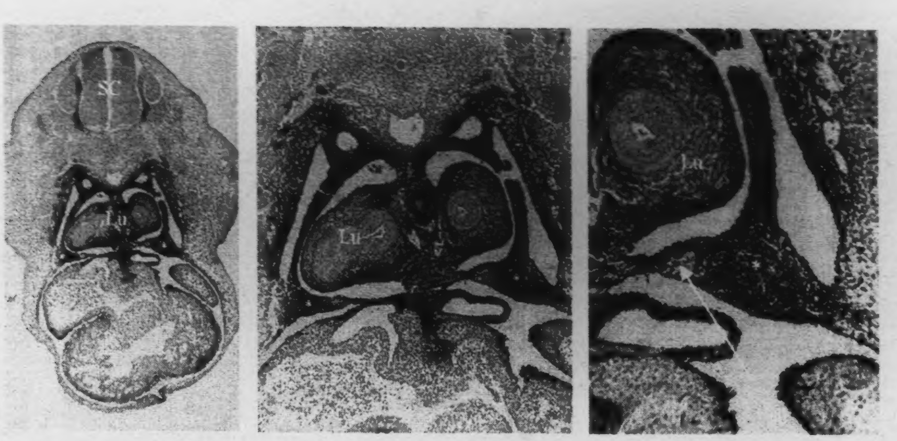


**Figure 5.4 Immunolabeling for RALDH2 in the primordial diaphragm, the pleuroperitoneal fold (PPF).**

There is intense RALDH2 expression in bilateral PPFs (*), as shown at different magnifications. Lu – lung; SC – spinal cord; Arrow – phrenic nerve.

## *5.4* ***DISCUSSION***

Nitrofen, bisdiamine, SB-210661 and 4-biphenyl carboxylic acid all induce diaphragmatic defects similar to those observed in infants with CDH. Importantly, all of these compounds inhibit RALDH2, a key enzyme responsible for the generation of retinoic acid from retinal. These data, in conjunction with that from past studies, support the hypothesis that perturbations of the retinoid system could be involved in the etiology of CDH.

Each of the four compounds induced diaphragmatic defects in the posterolateral corners of the diaphragm, similar to what is observed in infants with CDH. The differences in the number of treatments required for each compound is likely due to the length of time that each remains in the body before degradation or elimination. Nitrofen persists at elevated levels for at least three days (Choy et al., 1999), which would explain why a single large dose is all that is needed to induce diaphragmatic hernias. Conversely, biphenyl carboxylic acid may be much more labile (the parent compound, AH23848, has a plasma half-life of 1-1.5h), so that supplemental doses are necessary to produce sufficiently high concentrations to be effective. SB-210661 and biphenyl carboxylic acid may have a comparable half-life within embryonic tissues because the same dosages administered over the identical period of gestation produce similar fetal consequences.

In addition to inducing diaphragmatic defects, all four compounds can produce cardiac defects. These include maldevelopment of the outflow tracts of the heart and septal defects. The cardiac defects may result from problems with neural crest proliferation and differentiation in the fetuses (Sutherland et al., 1989; Taleporos et al.

1978; Solomon et al., 2000; Choy et al., 1999; Yu et al., 2001). There is no strong evidence, however, that diaphragm embryogenesis has any dependence on neural crest development. It may be that these compounds also interfere with the proliferation or differentiation of mesenchymal cells. The hypothesis that a defect in the formation of the mesenchymal substratum of the primordial diaphragm, the PPF, is associated with teratogen-induced CDH is being examined.

Recent studies using mice with a LacZ reporter gene linked to the activation of retinoid response element (RARE) demonstrated that nitrofen was inhibiting some aspect of retinoid function (Greer et al., 2001). The nitrofen-induced suppression of RARE was reversed by the addition of retinoic acid supplementation. Further, binding studies suggested that nitrofen was acting upstream of the binding of retinoic acid to its receptors. The preceding step in the retinoid cascade is the NAD-dependent oxidation of all-*trans*-retinal to all-*trans*-retinoic acid by RALDH2 (Niederreither et al., 1999). One of the CDH-inducing teratogens, bisdiamine, is a known inhibitor of alcohol dehydrogenase. Thus, it is hypothesized that the compounds were acting by interfering with that aspect of retinoid function. The results demonstrating that all four CDH-inducing compounds inhibit RALDH2 in a dose-dependent manner strongly support the hypothesis. Further, the most potent inhibitor of RALDH2, bisdiamine, was also the most effective at inducing diaphragmatic defects in embryonic rats. The other CDH-inducing compounds had similar dose-response curves for inhibition of RALDH2 activity. The concentration of nitrofen present in the embryo in response to gavage feed of 100 mg has been estimated to be in the μmole range. Similarly, exposure of fetal mouse embryos maintained in culture to ~15 μmole nitrofen produced a pronounced suppression or RARE-LacZ activation (Greer et al., 2001). The data in Figure 3 demonstrates that the $ED_{50}$ for nitrofen suppression of RALDH2 activity is within a similar range.

Collectively, the data support the hypothesis that the primordial diaphragm tissue, the PPF, is dependent on retinoid mediated signalling for its proper formation. Retinoic acid receptors function as transcriptional activators that modulate the expression of developmentally regulated genes by binding as a ligand/receptor complex to DNA sequences designated as retinoic acid response elements (Giguère, 1994). Consistent with this notion is the fact that the cervical mesenchymal tissues and developing diaphragmatic tissue strongly express proteins associated with the metabolism and binding of retinoids (Giguère, 1994; Båvik et al., 1997; Berggren et al., 1999). Further, retinoic acid has been shown to be involved in regulating extracellular matrix formation, mesenchymal cell migration, and establishment of dorsoventral polarity, all of which could be key components of early diaphragm embryogenesis (Ross et al., 2000; Yan and Sinning, 2001). However, a determination of the specific cells responsible for the embryological origins of the PPF will be necessary prior to ascertaining the role of retinoid signalling and its perturbation by CDH-inducing teratogens.

In ~60-65% of cases in infants with CDH there are no obvious associated anomalies other than the diaphragm defect (Fauza and Wilson, 1994; Losty et al., 1998). The reason that the diaphragm might be particularly susceptible to perturbations of the retinoid system is unclear. Immunolabeling for RALDH2 and HPLC analyses demonstrate that there are gradients of retinoic acid levels within the cervical mesenchyme (Ross et al., 2000; Yan and Sinning, 2001). Further, the levels of retinoic acid and associated activation of RAREs necessary for regulating retinoid-mediated transcription varies amongst genes in a dose-dependent manner (Ross et al., 2000). It is conceivable that the 'safety margin' for retinoic acid mediated regulation of primordial diaphragm development is relatively low and thus more susceptible to perturbations compared with other tissues. Heart development is also particularly susceptible, as cardiac anomalies are observed in the rat model of nitrofen-induced CDH (Migliazza et al., 1999a; Migliazza et al., 1999b) and are the most commonly associated defect in infants with CDH (Fauza and Wilson, 1994). RALDH2 is expressed in the developing lung (Giguère, 1994) and its suppression by nitrofen could explain some of the abnormalities of lung development observed in the nitrofen model of CDH (Nakao and Ueki, 1987; Wickman et al., 1993; Guilbert et al., 2000; Acosta et al., 2001).

Several hypotheses regarding retinoid system malfunction and the etiology of CDH in humans could be formulated. Transient deficiencies or imbalances in the levels of retinoid metabolites, binding proteins, nuclear receptors or retinoid metabolizing enzymes within the developing primordial diaphragm at the time of initial malformation of the PPF (approximately 4-5 weeks of gestation) could account for diaphragmatic defects and associated with CDH. These defects, in theory, could be due to acute dietary deficiencies, impaired placental transport, spontaneous malregulation within the retinoid metabolic cascade, teratogenic-mediated insults or chromosomal defects. Evidence for the latter arises from several references to an association of CDH with chromosome 15q defects (Kristoffersson et al., 1987; Smith et al., 2000; Bettelheim et al., 1998; Aviram-Goldring et al., 2000; Schlembach et al., 2001). A search of available databases found that some of the genes on chromosome 15 in the region of the deletion or translocation (15q24-26) encode for cellular retinoic acid binding protein-1 (CRABP-1). A nearby region (15q21.1) contains the gene encoding RALDH2, which emphasizes the importance of chromosome 15.

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# CHAPTER 6. GENERAL DISCUSSION

## *6.1* GENERAL DISCUSSION

The diaphragm is the principal inspiratory muscle. Upon inhalation, the diaphragm contracts, extending downward; the external intercostal muscles shorten, pulling the ribcage outward and upward, expanding the volume (vertical dimension) of the thoracic cavity, and resulting in an intrapleural pressure decrease relative to atmospheric pressure. The intra-alveolar pressure of the lung is decreased as well. This causes air to flow into the lungs until a new equilibrium with the external atmosphere is reached (Hooper and Harding, 2001). Although diaphragm function is not required for survival (Alcorn et al., 1980; Yu, 1998), it is crucial for proper development of the lungs (Sillos et al., 1988). However, important aspects of the embryology of the diaphragm have not been systematically studied and much of the current dogma may be incorrect. An understanding of diaphragm development also has significance in terms of better elucidation of the pathogenesis and etiology of congenital diaphragmatic hernia, a relatively common birth defect having an incidence similar to that of cystic fibrosis (1:2500-3000 Caucasian births; Collins, 1992; Hamosh et al. 1998). The majority of research in the CDH field is directed towards the understanding and treatment of the secondary consequences of CDH, such as pulmonary maldevelopment, rather than determining the reason for the primary insult, the hole in the diaphragm. Findings from this work provide information that will be a significant improvement to understanding diaphragm development and its malformations as well as providing the foundation for further research directed towards finding the mechanism underlying the pathogenesis of CDH in the model and possibly developing treatments based on the cause of the diaphragm malformation rather than the effects of the defect. The major findings from the studies described in Chapters 3-5 and Appendix 1 are discussed in this chapter.

## *6.2 EMBRYOLOGICAL ORIGINS OF THE DIAPHRAGM*

### *6.2.1 Source of the muscle cells forming the diaphragm*

Literature accounts of the formation of the diaphragm muscle attribute its composition to four separate sources. The use of antibodies for specific molecular markers of migratory muscle cells (Pax3/7, MyoD, myogenin) was used to identify muscle progenitor cells in the primordial diaphragm. The precursors populate the PPF at E12.5 and start to proliferate. The muscle precursors colonize the central axes of the dorsolateral costal, sternal costal and crural axes of the diaphragm, and then spread mediolaterally in the costal part, and dorsoventrally in the crural regions. By E17, these cells populate all areas of the diaphragm that will be muscularized. Results here show no evidence of contributions by the lateral body wall, dorsal mesentery or septum transversum to the diaphragmatic musculature, as suggested by current literature. The major limitation of this study is that it does not unequivocally identify the source of the muscle precursors (i.e. the cervical somites as hypothesized). This point will be addressed in Future Directions.

### *6.2.2 Origin of the crural muscle of the diaphragm*

These studies provide evidence that the crural region of the diaphragm has the same embryonic origin as the costal muscle. It has been postulated that the crural muscles are axial muscles and are derived from the same source as the muscles of the body wall (Langman, 1977; DeTroyer et al., 1983). The labeling experiments show no evidence that the muscle cells of the body wall colonize the diaphragm. Rather, the precursors of the caudomedial region of the PPF appear to form the crural parts. Stronger support for the non-axial origin comes from the finding that the c-met null mutant mice have an amuscular membrane where the diaphragm would form in a normal animal. These mice lack muscle derived from migratory muscle cells, but possess normal axial muscles. If the crural muscles were axial in origin, they theoretically should have formed in these embryos.

### *6.2.3 Relationship between phrenic axons, muscle precursors and myotubes*

This work has clarified some issues regarding the interaction between the developing nerve and muscle formation. It has long been a question whether the growing nerve axons precede, follow or develop simultaneously with the muscle precursors. Data obtained here show that during diaphragm development, the growth cones of the phrenic nerve lag behind the migratory muscle precursors by ~200 μm after the nerve contacts each hemidiaphragm at a central point, adjacent to the inferior vena cava on the right side (Allan, 2000). Myotubes appear to form ahead of contact with the phrenic nerve. In the developing diaphragm, the muscle cells show a greater density along the center of the axes of the diaphragm, around the nerve branches. This is in agreement with findings of Allan and Greer (1997) who showed a clear correlation between phrenic nerve intramuscular branching and myotube formation. These primordial muscle cells proliferate and begin to differentiate from the central point outwards. MyoD-expressing cells continue to proliferate and differentiate along the primary myotubes at E17 in preparation for secondary myogenesis.

This does not answer some fundamental questions such as what factor lures the myoblasts to the PPF, why the phrenic nerve always enters the muscle at the same point, and how the branches determine which muscle fibers in what part of the diaphragm to innervate? It has been shown that the phrenic nerve in the rat has an ordered somatotopic innervation pattern wherein the rostrocaudal position of the phrenic motoneuron cell bodies in the cervical spinal cord are faithfully represented in the diaphragm (Laskowski et al., 1991). The phrenic motoneurons derived from C3-C5 innervate the costal muscle of the hemidiaphragms in a rostrocaudal fashion. The ventral-most region is innervated by C3 while C4 and 5 dominate the dorsal portion (Laskowski and Sanes, 1987). After contacting the diaphragm, the phrenic nerve divides into rostral and caudal branches by E15. The caudal branch further divides, forming caudal and crural branches. The motor axons preferentially enter the appropriate branch to innervate their targets. Questions remain about the guidance cues that attract the phrenic nerve to one particular initial point of contact and how the axons enter the proper branch of the phrenic nerve (Laskowski and Sanes, 1987). It is not known whether these actions are due to positional cues in the muscle, some form of local axonal guidance at the bifurcation point of the nerve, axonal ordering, or some other factor. The number of motoneurons is matched to available muscle fibers by apoptosis (Landmesser, 1992).

### *6.2.4 Implications for CDH Studies*

There are numerous theories regarding what may go awry during development of the diaphragm, leading to CDH. One possibility that has been suggested is that the hole results from a problem with muscle formation, specifically that fewer muscle progenitors are available to form the diaphragm, resulting in a weak sheet of muscle that is easily ruptured (Skandalakis et al., 1994). From this study, it appears that the muscle precursors migrate, proliferate and differentiate in the animal model of CDH in the same way as observed in control animals. The most striking difference is the tissue defect seen in the PPF of nitrofen treated animals and this has become the focus for the investigation of the pathogenesis of CDH. The molecular composition of this structure needs to be elucidated, but this point will be discussed later.

### *6.2.5 Future Directions*

A basis has been established for further investigation into the process of diaphragm development to address some other unanswered questions. It is hypothesized that the diaphragm, like the fore limb muscles, is formed from muscle precursors that originate from the cervical somites. Myogenic precursors migrate from the somites and travel to the presumptive limb. A subpopulation is predicted to continue migration to the PPF. Experiments need to be performed to identify the origin of the muscle precursors that populate the PPF. It is possible to inject individual somites with a fluorescent tracer, DiI, to label the cells. The fate of these cells could then be determined as they migrate by observing serial sections at sequential ages following injection. This has been successfully used to map somitic cells in the rat (Lee and Sze, 1993) and chick (Hayashi and Ozawa, 1991; Liu and Bagnall, 1995). This technique could be utilized in whole-embryo culture. Performing these labeling experiments in rat whole-embryo culture would give more temporal control over the cell labeling. It takes the cells approximately two days to reach the brachial plexus from the somites. However, in the embryo culture, 48 hours of development *in vivo* equate to 36 hours in culture. It may be possible to time the injection of DiI so that soon after the dye has been injected, the cells start to migrate from the lateral aspect of the somite. Theoretically it might be possible to detect labeled cells right after the presumed population leaves the level of the limb bud and proceeds to migrate towards the PPF. A potential problem with this is that the dye, which incorporates into the plasma membrane, is transferred to daughter cells with proliferation, and the dye intensity becomes diluted with successive cycles of replication (Bagnall, 1992). If the migratory cells replicate as they migrate, the DiI may not remain detectable long enough for their ultimate destination to be unequivocally determined. It is hypothesized that the cells do not enter the replicative phase until reaching the end point of their journey, so dye labeling may be the simplest method. Alternately, to follow the somitic cells during their migration, an adenovirus expressing the green fluorescent protein (GFP) could be injected into the somites to transfect the cells and then detected in serial sections of fetuses at different gestational ages. The adenovirus vectors have been successfully injected into E10 embryonic mouse hearts and expression of GFP was detected in embryonic heart tissue (Christensen et al., 2000). This technology is available through the Perinatal Research Center in the laboratory of Dr. Jason Dyck. However, with this method, all cells of the somite would be transfected with the virus, potentially complicating detection of the myogenic precursors, and if the titer of virus particles used was too large, other cells outside of the somite of interest could be affected (J. Dyck, personal communication). The amount of virus injected would have to be carefully controlled to avoid this problem. To determine the concentration would probably require a number of trials, since the efficiency of transfection is cell type-specific. The surgical manipulations necessary for injecting the somites of the embryos would be very delicate and would require a high degree of skill, but Dr. Dyck considers an experiment of this type to be feasible. This difficulty may be avoided by using a muscle or migratory cell-specific promoter such as the *c-met* promoter to drive expression of the GFP. This would also be useful to determine the number of precursors that initially migrate to the PPF. If only 30-100 cells leave the somite (Megeney and Rudnicki, 1995), how many of those migrate into the limb bud or continue onward to the PPF?

Another aspect of diaphragm development that warrants further investigation is the identification of the factors expressed along the migratory route of the muscle precursors in their migration from the somite to the PPF. The molecule HGF/SF has been identified as the ligand for the c-met receptor on migratory cells, as well as acting as a chemoattractant for motorneurons. It is likely that this molecule functions to guide the muscle precursors, but whether this factor is expressed constitutively along the migratory path, only by specific cells, or if it forms a gradient is not known. To achieve this aim, immunohistochemical labeling for HGF/SF could be done. If a suitable antibody for HGF/SF is not available, *in situ* hybridization can be done.

A project related to diaphragm formation is underway utilizing the Fgf10 knockout mice discussed in Chapter 4. The null mutants show lung agenesis, but in addition they completely lack fore- and hind limbs. This begs the following question: what happens to the muscle precursors that migrate to invade the developing limb? Do they undergo cell death without a target, do they lose the migratory phenotype and become axial muscle cells contributing to the body wall musculature, or do they respond to guidance cues from the PPF and populate the developing diaphragm? The hypothesis that the limb precursors contribute to the diaphragm is supported by a preliminary observation that the diaphragms of FGF-10 nulls appear thicker than those of heterozygous of wild type littermates. Counts of the muscle fibers have to be done to ascertain if there is a greater density. To answer these questions, similar techniques to those discussed previously for determining the origin and destination of the muscle precursors migrating from the somite can be used. Alternately, serial sections of embryos aged E10 to E13 could be labeled for muscle specific factors (MyoD, myogenin) and migration specific factors (Pax3, c-met) to identify the muscle precursors and to localize sites of differentiation. TUNEL, combined with a DAPI nuclear labeling assay, can be performed on these sections as well to see if there is increase programmed cell death at the level of the limb buds. Diaphragms from animals E14 and older would be isolated, labeled or counterstained, then sectioned to determine if the muscle of the diaphragm is significantly thicker or if the muscle fibers have a higher density of distribution.

## *6.3 INSIGHT INTO THE PATHOGENESIS OF CDH IN THE ANIMAL MODEL*

The pathogenesis of CDH remains an unanswered question in the research field. It is hypothesized that the defect in CDH in the rodent model is due to a malformation in the primordial diaphragm, the PPF (Greer et al., 2000). The data discussed in Chapter 3 refute the idea that the muscle defect is due to a problem in muscle precursor migration or proliferation. The current thinking is that the defect occurs because the mesenchymal substratum does not provide a complete foundation for muscle formation. It has been found using c-met null mice that the defect observed in CDH can be induced in the absence of myogenic processes. This supports the hypothesis that the amuscular component of the diaphragm underlies the pathogenesis of CDH in the animal model, shifting the focus of studies of CDH to an earlier developmental stage than previously considered.

Another common hypothesis in the field of CDH is that a primary defect with the developing lung is the basis for the pathogenesis of CDH (Iritani, 1984; Cilley et al., 1997). Fgf10 knockout mice, displaying lung agenesis (Sekine et al., 1999) have been used to investigate this. The defect characteristic of CDH can be induced in these mice. This refutes the idea that the lung problems cause CDH in the model, as no or minimal lung tissue is present in these animals. These findings strengthen the hypothesis that CDH results from a defect in the mesenchymal tissue forming the PPF.

### *6.3.1 Future Directions*

If the defect in the CDH model is not the result of a lung defect, muscle formation problems (Babiuk and Greer, 2002), phrenic nerve development or closure of the pleuroperitoneal canals (Allan and Greer, 1997), then the pathogenesis must be founded elsewhere, such as the PPF (Greer et al., 2000). The defect in the PPF is poorly understood. The molecular composition of the normal PPF is not known, nor are the molecules it may express or secrete, let alone that of the missing region. Experiments that are designed to identify the components of the normal PPF and what may be absent or restricted in expression in the defective PPF are underway. A proteomics project to determine the molecular make-up of the amuscular membrane found in the c-met nulls has been initiated. Proteomics is a study of the protein complement of a cell, with the proteome being the full array of proteins expressed in a particular cell or tissue at a specific developmental time or state (i.e. healthy or diseased). Amuscular membranes and central tendons from control diaphragms are being isolated for study. The control tissue is being used to determine the best extraction method and to see how many proteins are expressed in the non-muscular part of the diaphragm. Each sample or a pooled group of tissue will be ground, then homogenized with a buffer to extract proteins from the tissue. These protein extracts will be subjected to 2-dimensional electrophoresis to separate the proteins, where in the first dimension proteins are separated by molecular weight and in the second dimension the migration is dependent on charge or the isoelectric point (pI). The gels will be silver-stained to visualize the protein profile, de- and restained with Coomassie Blue, and spots representing proteins of particular interest will be excised and mass spectrometric identification will be performed by the Institute for Biomolecular Design (IBD) at the University of Alberta. Preliminary results from control diaphragm central tendon samples have been obtained. The number of protein spots is in the range of 100-200 spots according to Dr. G. Sawicki, with approximately 20 in high abundance. Tentative identification of the spots based upon molecular weight will be made and a small number will be analysed further by IBD.

An identification of structural proteins in the amuscular membrane will be used to determine antibodies that can be used to stain the normal and defective PPFs for identification of major proteins composing the PPF. Also, once the components have been identified, it would be possible to determine which proteins are products of

RA-responsive genes or are directly influenced by RA-mediated processes. There may be knockout mice available for these genes. Such mutant lines would make it possible to investigate the roles of the specific genes in formation of the PPF. Fetuses at ages during which the PPF is forming would be isolated, sectioned and stained. If the PPFs in this tissue were defective, it could correlate significantly with the target of the teratogens.

The second project is partially complementary to the proteomics. Robin Clugston, a fellow graduate student, will use laser capture microdissection (LCM) to isolate single cells from this tissue structure. mRNA will be isolated from these cells and RT-PCR will be performed to identify genes that are predicted to be expressed in the PPF, including HGF/SF. Probes complementary for the RNA of proteins identified by proteomics methods will be used for RT-PCR to see if expression of those genes is reduced by treatment with nitrofen or the other teratogens.

## *6.4 FINDINGS REGARDING THE ETIOLOGY OF CDH IN THE ANIMAL MODEL*

CDH has been studied for decades using the nitrofen model in rodents. The work in this thesis has shown that three other compounds can cause the same diaphragmatic defect in rats. The compounds 3-biphenyl-carboxylic acid (BPCA), SB-210661, and bisdiamine when administered early in gestation on several subsequent days induce posterolateral defects that are identical to those seen in the nitrofen model in fetal rat diaphragms. Another compound related to BPCA called biphenyl methanol also induced hernias, but with a significantly lower incidence. For BPCA and SB-210661, this work represents the first systematic characterization of their effects on diaphragm development in the rat. There have been only single publications for each compound that briefly mentioned the observation of diaphragm defects (Solomon et al., 1989; Sutherland et al., 2000). Bisdiamine has been studied quite extensively in Japan in this context (Tasaka et al., 1992; Hashimoto et al., 1998), but these data are little known outside of Japan.

The differences found in the number of doses necessary to induce defects probably reflects, in part, variations in the half-life of the compounds in the body of the rat. It was hypothesized that bisdiamine and the other compounds being investigated acted to inhibit retinoic acid production. The fact that bisdiamine potently inhibited spermatogenesis (Coulton et al., 1960; Hershberger et al., 1969) which is a process dependent on retinoic acid, strengthens this hypothesis. Jörg Mey of Aachen University was recruited for collaboration on this project to test the four compounds. In a cell assay system, it was found that all four compounds that cause diaphragmatic defects strongly inhibit production of retinoic acid. Evidence for the possible involvement of the retinoid system in CDH has been obtained. However, it does not prove that inhibition of retinoic acid synthesis is the main or sole point of interaction of the compounds with the retinoid system. This work does not correlate strongly with human CDH in that, as stated in the General Introduction, these teratogens have not been linked to human CDH (Torfs et al., 1992), while other unknown environmental factors or compounds could be responsible. Also, the inhibition of RALDH2 examines only the final step in the retinoic acid synthesis pathway. The finding from this study was that all four teratogens inhibit the synthesis of retinoic acid directly but not necessarily exclusively.

### *6.4.1 Future Directions*

More work should be done to identify specifically the point(s) of action of the teratogens in retinoid metabolism. The final step in RA production catalysed by retinaldehyde dehydrogenase 2 is inhibited, but considering that bisdiamine is known to inhibit alcohol dehydrogenase, it is conceivable that the conversion of retinol to retinal is inhibited. Other earlier steps in the pathway may be compromised, as well. The initial point of the insult to the retinoid system caused by the compound has to be identified to understand the etiology of the defect in the animal model.

Some preliminary data have been gathered about the utility of retinol or retinoic acid supplementation in prevention of lung and diaphragm defects induced by nitrofen. It has been shown that retinol administration prior to, with or after nitrofen administration does reduce hernia incidence and lung hypoplasia in rats (Thébaud et al., 1999, 2000; Babiuk, unpublished results). There is a reduction, not a complete abolition of the effects of nitrofen, possibly due in part to the timing of administration. Retinoic acid rescues some defects caused by retinoic acid depletion or genetic defects in embryos (Dickman et al., 1997; Pasqueletti et al., 2001). Preliminary results gathered have not shown success in the use of retinoic acid treatment in reducing the incidence of hernias in the nitrofen model, but RA does appear to lessen the lung hypoplasia seen in the fetuses. It is possible that the retinoic acid used for treatment does not efficiently enter the fetal circulation with maternal administration. This can be tested using the LacZ-RARE mice, which are being bred in the animal facility. Treatment of pregnant transgenic mice with the teratogenic cocktails as described in Chapter 4 and analysis of LacZ expression (decreased staining) in the fetuses will indicate if the retinoid system is perturbed *in vivo* by these drugs. If the transgene expression is restored after maternal retinoic acid administration, the retinoic acid is effectively delivered to the fetuses.

Retinoic acid is not the only active retinoid with physiological importance. The visual system for example requires retinal, not retinoic acid. If diaphragm formation is not (wholly) dependent on retinoic acid but requires another active metabolite of retinol for certain aspects, this could explain the ineffectiveness of retinoic acid in this paradigm and only partial success of retinol in preventing CDH. Another more likely explanation is that retinoic acid is much less effective at transferring across the placenta to the fetus, as has been shown (Shukla, et al., 1986). Future work will be designed to determine exactly what steps in the retinol pathway are affected by the teratogens, as well as what retinoid(s) are critical for diaphragm formation.

A collaborative project is underway with the laboratory of R. Blomhoff in Oslo that is anticipated to provide quantitative data on levels of retinoids and related molecules (i.e. RBP) in control rats and those treated with nitrofen. Major et al. (1998) reported that the levels of retinol and RBP in the plasma of infants born with CDH were lower than in normal infants. These levels were found to be higher in mothers of infants with CDH when compared with levels in mothers having healthy babies. This is suggestive of impaired transport of retinol from the maternal to fetal circulation and/or impaired retinoid metabolism in the fetuses. Such a reduced level of retinol would result in lower retinoic acid and other retinoid concentrations that are hypothesized to be important in normal diaphragmatic development. Embryos staged from E10.5 to E13.5 have been isolated from nitrofen and control dams. Plasma and liver samples have been collected from the dams as well. All samples have been sent to Oslo where retinoids and carrier proteins will be isolated and HPLC measurements will be performed. The hypothesis is that values for retinoid concentrations in the fetuses isolated from nitrofen treated animals will be lower than those in control fetuses.

In the nitrofen model, the PPF has been shown to be defective in some fetuses from treated animals (Greer et al., 2000). The missing piece of the PPF appears to correspond to the location where the missing part of the diaphragm is observed. This is the basis for the hypothesis of the pathogenesis of CDH in the model that much of this thesis is based upon. To show that BPCA, SB-210661 and bisdiamine act through the same mechanism as nitrofen in causing CDH, it is important to treat rats with these three compounds, isolate and section the fetuses to determine if similar PPF defects are induced. The inhibition of RALDH2 by all compounds and data showing that all induce similar defects in the PPF would support the hypothesis that CDH in the rodent model is the result of a defect in retinoid metabolism, which disrupts a process necessary for the mesenchymal substratum to form.

There is a colony of RARE-LacZ transgenic mice presently being bred by Laboratory Animal Services at the University of Alberta. These animals have the beta-galactosidase gene-coding region under the control of a retinoic acid response element-containing promoter. BPCA, SB-210661, bisdiamine and nitrofen will be given to pregnant mice on E8 in experiments similar to ones performed by J. Greer

(Greer et al., 2002) to investigate whether all of the compounds can reduce expression of the transgene as has been shown with nitrofen.

These mice will be used to determine the period during development when the RARE-LacZ gene is turned on or shows increased expression in the primordial diaphragm. This would correspond to the period of highest susceptibility to the teratogens and decreased retinoic acid production. This information will make it possible to employ conditional mutants (animals in which a particular gene can be "turned on/off" at a specific time) for retinoic acid-responsive genes to show that the defect in the PPF directly results from problems with the retinoid system. If it is possible to obtain a conditional mutant for a gene hypothesized to be important in the pathogenesis of the defective PPF, the gene could be silenced during the developmental window when RA production/concentration is critical for PPF development. Turning off expression of the gene at this time would result in the same type of tissue defect seen in the nitrofen model of CDH. Evidence would then be obtained for the mechanism or the candidate genes that might underlie the pathogenesis of the defective PPF.

## *6.5 CHARACTERIZATION OF OTHER MODELS OF CONGENITAL DIAPHRAGMATIC DEFECTS*

Diaphragmatic hernia has not been linked to genetic defects in all cases. It is seen as a malformation in numerous syndromes that do have a genetic component (Cunniff et al., 1990; Pinar et al., 1994; Choo et al., 2002) or as the result of specific chromosomal abnormalities (Kristoffersson et al., 1987; Bettelheim et al., 1998;

Aviram-Goldring et al., 2000; Smith et al., 2000; Schlembach et al., 2001). Bochdalek hernias are studied in rodent models created by administration of teratogenic compounds or by surgical means in other animals, but other types of diaphragmatic malformations are much less researched, as good models are not available.

Two genetic mouse mutant lines with diaphragm malformations have been partially characterized as part of this thesis (Appendix 1). Some *Slit3* null mutants have a congenital diaphragmatic hernia as part of the null phenotype. This hernia resembles the central transversum hernia. The *Fog2* mutants have a partially muscularized diaphragm without a specific herniation. This situation resembles the eventration type of diaphragm defect (Stokes, 1991).

### *6.5.1 Future directions*

Both types of diaphragm malformation arise from defects in genes that previously were not known to be important for diaphragm development. However, as both result in diaphragm defects, one appearing to be non-muscle specific and the other predominantly muscle specific, these mice could provide models for diaphragm formation. More detailed analysis of the genetic pathways affected by these mutations could be informative about the genes that regulate diaphragm embryogenesis. However, dissection of this genetic control is in the domain of those labs that developed the lines and have the capability to perform such studies.

There is another knockout mouse line in which diaphragmatic defects result, namely the *WT1* (Wilms' tumour suppressor gene) knockout animal. In the homozygotes, the kidney and gonads do not develop. There are also mesothelial, heart and lung defects (Kreidberg et al., 1993). The *WT1*$^{-/-}$ fetuses die between days 13 and 15 of gestation, likely due to these defects rather than the kidney defects. It was found in fetuses at E 14.5 that the diaphragm had not fully formed, leaving communication between the thoracic and abdominal cavities. These animals had posterolateral defects, similar to the position of the holes seen in the nitrofen model. *WT1-LacZ* transgenic fetuses were examined in the Greer laboratory, sectioned and stained for beta-galactosidase expression. The expression domain of the *WT1* gene product is in the mesothelial lining of the abdominal cavity; as well, *WT1* shows strong expression in the dorsal region of the PPF, a region which corresponds to the region observed to be defective in the nitrofen model of CDH (Greer et al., 2000), suggesting that there may be a connection to the etiology of CDH in the model. These data correspond with the findings showing *WT1* expression in the coelomic epithelium, developing limb, septum transversum and epicardium (Moore et al, 1998).

*WT1* plays a role in epithelial to mesenchymal transformation. It is also believed to be involved in differentiation of certain tissues. Structural analyses have shown that it has homology to known transcription factors, and it has been shown to possess DNA binding capabilities and can act as a transcriptional regulator of a variety of genes. The WT1 protein interacts with other proteins, some of which are transcription factors or act to regulate gene activity, and serves to modulate their activity (Scharnhorst et al., 2001). These multiple functions for WT1 suggest many levels of complexity are involved, and the reason for the observed diaphragmatic defects may not be as simple as a mutation in the *WT1* gene.

However, defects in *WT1* itself do not seem to have a role in human CDH at least. A study of children born with CDH was prompted by the data published by Kreidberg et al. (1993). 27 CDH victims were screened for *WT1* mutations, but no defects in the gene were found. There is a single report of a *WT1*-associated syndrome, Denys-Drash Syndrome, in which a diaphragmatic hernia was observed (Devriendt et al., 1995). The defect is probably not in the Wilms' tumour 1 gene, but instead lies either upstream or downstream in the signalling pathway. The gene product is a transcription factor, which has a large number of potential target genes, including itself, retinoic acid receptor alpha and the extracellular matrix components syndecan and E-cadherin (Scharnhorst et al., 2001). *WT1* has been shown *in vitro* to activate syndecan-1, which functions with other receptors to promote the translation of extracellular signals into intracellular actions and is important in the mesenchymal to epithelial conversion and the differentiated phenotype of epithelial cells.

A connection to the retinoid hypothesis in the etiology of CDH can be formulated for *WT1*. Some of the target genes for *WT1* are putative retinoic acid responsive genes. Decreased retinoic acid production could affect the expression of these targets, decreasing the function of the WT1 protein. To determine if this is an important factor in causation of the diaphragm defects in *WT1* knockouts, greater understanding of its gene targets and their possible relationship with the retinoid system is needed. Retinoic acid could have a more direct role in the pathogenesis of the defects seen in these mutants. It was found *in vitro* that differentiation of carcinoma cells and embryonic stem cells with retinoic acid triggered *WT1* expression and stimulated its DNA binding activity (Scharnhorst et al., 1997), whereas DMSO-induced differentiation resulted in decrease expression. Expression of insulin-like growth factor 1 receptor, a putative WT1 target, was increased in response to *WT1* activation, and the expression was seen in endodermal, glial and epithelial cell types (i.e. tissue-specific). These findings indicate that retinoic acid may be important in activation of *WT1* expression and that in the teratogen model where retinoic acid production is inhibited, reduced expression of *WT1* in the PPF may be an important factor, considering the consequences of the *WT1* knockout. It must be noted that these findings are from an *in vitro* culture system, and may not reflect the *in vivo* functions of *WT1*. Still, it provides an interesting basis for a hypothesis of the role of this gene in diaphragm formation.

Some fundamentally new insights on the process of diaphragm formation have been gained from the work described in the thesis. However, there are many questions left unanswered. Has the etiology and pathogenesis of CDH in the animal model been elucidated? No, but certain aspects of these have been clarified to an extent and have provided a foundation and a direction for more experiments and hypotheses have been provided. Is the problem of human CDH close to being solved? Again, no, but the data from the model do give an indication that an understanding of CDH will require a closer examination of the biochemical and metabolic status of pregnant women and their fetuses during the course of early pregnancy.

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# APPENDIX

Based on contributions to the following works

Ackerman, K.G, Herron, B.J., Rao, C., Huang, H., Epstein, J.A., **Babiuk, R.P.***, Greer, J.J., and Beier, D.R. (2002) An ENU-Induced Mutation in *Fog2* Results in Abnormal Diaphragm, Lung and Cardiac Development. In preparation.

Yuan, W., Rao, Y., **Babiuk, R.P.***, Greer, J., Wu, J.Y., and Ornitz, D.M. (2002) A genetic model for congenital diaphragmatic hernia created by a null mutation in the *Slit3* gene. In preparation.

*contributions to these works: isolation of diaphragm tissue from fetal mice of different ages, immunolabeling of diaphragm tissue, photographed diaphragms (unlabeled and labelled) and providing them to the primary authors

Assisted in writing of manuscript (provided methods details and discussion of diaphragm malformation)

These results have been included in the following:

Ackerman, K.G., Herron, B., Rao, C., Huang, H., **Babiuk, R.P.**, Kochilas, L., Epstein, J.A., Greer, J.J. and Beier, D.R. (2002) An ENU-induced mutation in Fog2 produces both primary pulmonary hypoplasia and abnormal diaphragmatic development in mice. American Thoracic Society Meeting. In press.

Ackerman, K.G., Herron, B., Rao, C., **Babiuk, R.P.**, Greer, J.J. and Beier, D.R. (2002) Abnormal diaphragmatic development and pulmonary hypoplasia caused by an ENU-induced mutation in mice. American Thoracic Society Meeting 306.

## ***INTRODUCTION***

The genetics of diaphragm development are poorly understood. Slowly the genes involved in the formation of the diaphragm in the mouse have been elucidated by generation of mutant mouse lines. For example, the *WT1* gene appears to be important for normal diaphragm development (Moore et al., 1998; Kriedberg et al., 1993). Also, phenotypic screening of mouse mutations is becoming a very powerful tool in identifying developmentally important genes (Anderson, 2000; Kasarskis et al., 1998).

In the Chapters 3-5, work based upon chemical models of diaphragmatic hernia in rodents was discussed. The studies described in those chapters investigated developmental malformations (i.e. CDH) of rat or mouse diaphragms that were the result of teratogen administration. However, the genetic control governing the formation of the diaphragmatic primordium is still not known. As discussed in the opening paragraph of this appendix, mouse mutants are becoming an important tool for identifying important developmental genes in many organ systems. As a result of this phenomenon, two groups contacted the Greer laboratory because of the expertise it has shown in the fields of diaphragm development and CDH research. The labs of D. Beier (Harvard) and D. Ornitz (Washington University in St. Louis) engineered genetically mutated mice as part of their own research. It was determined that the phenotypes of these mouse lines included diaphragmatic malformations. Collaborations were established to elucidate the pathogenesis of the diaphragm maldevelopment observed in these models.

Determination of the pathogenesis was accomplished by isolation of the diaphragms from mutant and control mice of different embryonic ages and immunolabeling the tissue for markers of nerve and muscle development. From these genetic models of aberrant diaphragm development and from further studies of the genes involved, insight into the genetic control of diaphragm formation can be gained and, possibly, into the genetic contribution to the human condition of CDH.

One of these genetic models is the *Slit3* knockout mouse. Slit is the ligand for the Robo receptor. *Slit1* and *2* are expressed primarily in the central nervous system, and have been shown to have functions in axonal guidance and as guidance molecules for other cell types in the mouse. No specific function is known for the Slit3 protein, but in contrast to Slit 1 and 2, Slit3 is expressed weakly in the CNS but strongly in some peripheral tissues. David Ornitz's laboratory in St. Louis generated these knockout mice to elucidate *in vivo* functions of *Slit*3. It was found that the null animals for this gene develop a herniation of the diaphragm (penetrance of the hernia phenotype – 68%), resulting in respiratory distress causing death in 24% of the nulls by day 40 and 58% by day 150 (Yuan et al., 2002).

The other mouse mutant described here has an induced mutation in the *Fog2* gene. David Beier at Harvard uses N-ethyl-N-nitrosourea (ENU) to induce novel mutations in mice, specifically recessive mutations that cause developmental abnormalities such as those seen in human congenital malformation syndromes (Herron et al., 2002). A mutation with phenotypic malformations of the cardiac and pulmonary systems and the diaphragm was identified. The mutated locus was found to be *Fog2* with a positional cloning strategy. This gene is known to be necessary for normal heart development. No role in diaphragm or lung development had previously been attributed to *Fog2* before the initial observation of these phenotypes by the researchers who developed the mutants.

## MATERIALS AND METHODS

### *Tissue Isolation*

The labs that engineered the mutant lines provided fixed fetal mouse samples. The diaphragms from control, heterozygous and null animals were isolated as previously described. Diaphragms were isolated from fixed fetal mice. Upon removal, diaphragms were washed for 30 minutes in PBS pH 7.4 prior to immunohistochemical processing.

### *Antibodies*

Antibodies were diluted in PBS containing 0.4% Triton X-100 (BDH Inc., Toronto, Canada). Muscle fibers were labeled with mouse MF 20 used at a dilution of 1:25. This antibody recognizes myosin heavy chain (MHC). Phrenic nerve labeling was performed at a dilution of 1:1 with mouse anti-neurofilament antibody 2H3, which detects the 165 kDa isoform of the protein. The secondary antibody was diluted to 1:200 with PBS with 0.4% Triton X-100. Biotinylated goat anti-mouse IgG (Sigma; whole molecule) was used for both primary antibodies.

### *Immunohistochemistry*

The diaphragms were placed in wells of a 96-well microtiter plate containing PBS. The PBS was removed from the wells by vacuum aspiration and replaced with PBS/0.4% Triton X-100 containing 0.3% $H_2O_2$ for 20 minutes. The solution was removed and PBS was added to wash the diaphragms for 2-3 minutes. Diaphragms were then incubated in 1:20 normal goat serum (Sigma) for 1 hour. After removal of the goat serum, diaphragms were incubated in diluted primary antibody MF 20 or 2H3 overnight at 4°C. After washing the diaphragms in PBS, tissue was incubated in secondary antibody for 1 hour at room temperature. This was followed by PBS washing and further incubation in a 1% avidin biotinylated-peroxidase complex (ABC; Vector Laboratories (Canada) Inc., Burlington, ON) at room temperature for 1 hour. Diaphragms were washed thoroughly with PBS. Antibody labeling was visualized with a DAB (3,3-diaminobenzidine tetrahydrochloride; Sigma) reaction product intensified with nickel (0.1M Tris-buffered solution pH 8.0 containing 0.04% DAB with 0.04% $H_2O_2$ and 0.6% ammonium nickel (III) sulphate). Labeling was detected at room temperature over 5-10 minutes. Mutant and control diaphragms were stained with this protocol. Diaphragms were photographed using a Nikon 990 digital camera mounted on a Leica research microscope, imported into Adobe Photoshop, contrast-adjusted and labels added.

## RESULTS

### *Slit3 null diaphragms*

The *Slit3*$^{-/-}$ diaphragms were observed to be abnormal at the ages examined. The pathogenesis of the central tendon first appeared at E14.5. An apparent thinning of the region of the central tendon retrosternally could be observed at E14.5 (4/4 fetuses). The central tendon was adherent to the liver at E14.5 and E15.5 (3/3 fetuses). At E17.5 (2/2 fetuses), it appeared that pressure from the growing liver ruptured this weakened area and liver tissue was forced through the resulting defect, forming a "plug" (Figure 1B). Upon dissection of the diaphragm (i.e. removing all liver tissue), the defect could be observed to be an oval, almost circular hole in the diaphragm, situated in the central tendon. This defect did not extend into the muscularized lateral portions of the diaphragm, as determined by labeling of the muscle fibers with the anti-myosin antibody. The phrenic nerve and its innervation pattern were normal in the null diaphragms when compared with the wild type. From observation of the lungs from the null animals, there did not appear to be any gross external pathology present, such as the hypoplasia seen with the nitrofen/teratogen models of CDH or in the human condition.

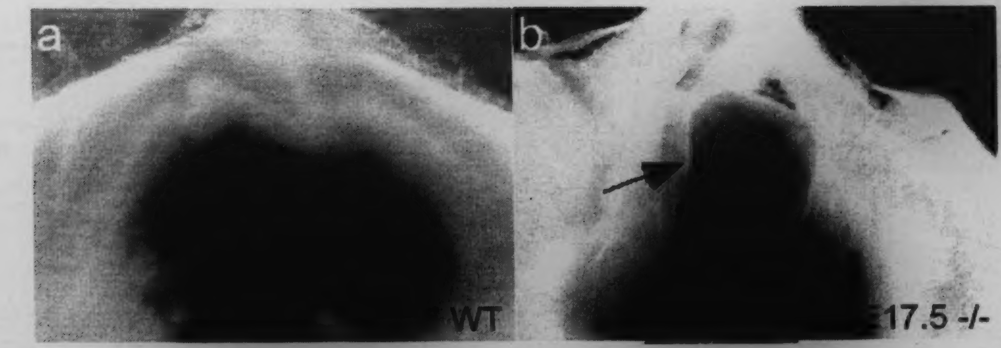

**Figure 1** CDH in *Slit3*$^{-/-}$ mice

(a) Normal E17.5 diaphragm with liver attached underneath. (b) E17.5 *Slit3*$^{-/-}$ diaphragm with a liver plug (arrow).

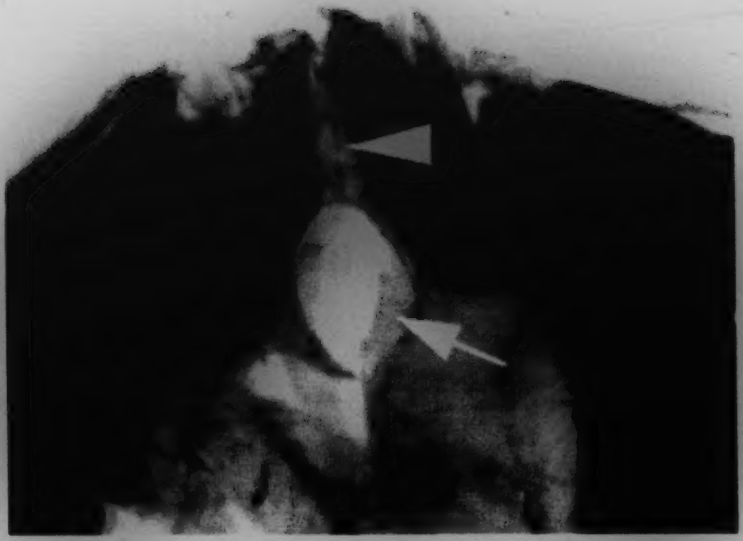

**Figure 2** E16.5 *Slit3*$^{-/-}$ diaphragm.

Whole-mount immunohistochemistry in E16.5 *Slit3*$^{-/-}$ diaphragm with MF-20 (anti-myosin, Hybridoma bank). Arrow: defect in central tendon, Arrow head: defect in muscle.

### *Fog2 mutant diaphragms*

Isolation of *Fog2* diaphragms from the mouse mutants was more difficult than the *Slit3*$^{-/-}$ mice. This was due to the very limited muscularization of these diaphragms that provides mechanical support to the diaphragm. Immunolabeling for myosin at E17.5 showed that the muscularized area of the diaphragms was restricted mainly to the medial region (Figure 3B,D) and the muscle fibers were distributed abnormally. The myotubes that were present did not appear to be oriented in the normal mediolateral fashion as seen in control diaphragms; rather they ran in a ventrodorsal pattern. A large portion of the dorsal and dorsolateral musculature was absent, leaving a thin, transparent non-muscular membrane that covered the surface of the liver. The phrenic nerve visualized by neurofilament labeling at E15.5 also showed an irregular branching pattern when it contacted the diaphragm (Figures 3F, 4) when compared with a normal diaphragm (Figure 3E). When the thoracic cavities of the mutants were opened, a significant degree of lung hypoplasia was seen. This was common to all mutants examined (4/4 E15.5, 4/4 E17.5 fetuses)

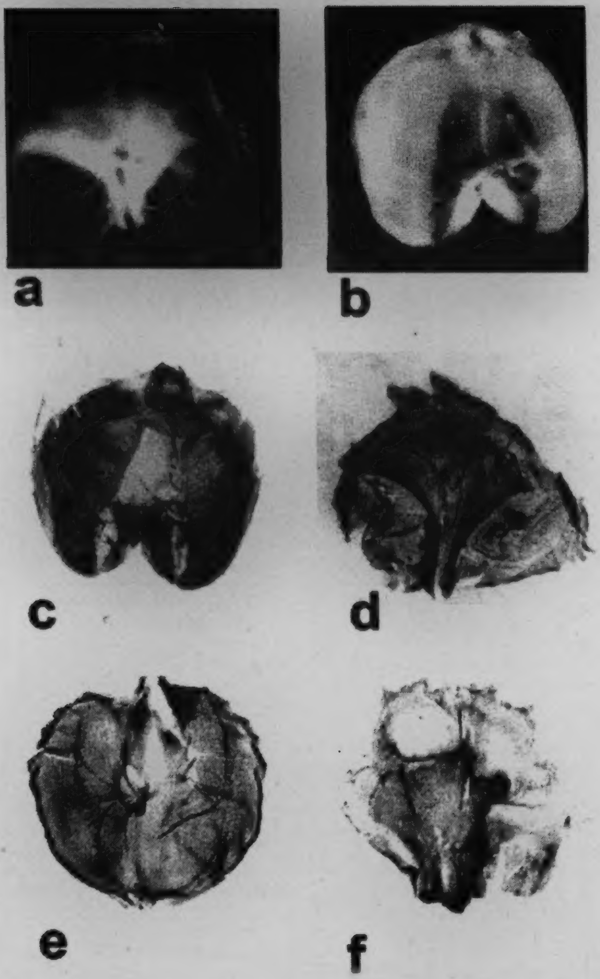

**Figure 3** **Diaphragm in *lil* mice has an abnormal pattern of muscularization**

Whole mount unstained diaphragms from E15.5 wildtype (a) and *lil* mutant (b) mice. In the mutant mouse, the diaphragmatic tissue is intact, but lacks apparent muscularization in the dorsal regions. Whole mount diaphragms labeled for myosin (MF-20) from E17.5 wildtype (c) and mutant (d) mice. The mutant diaphragm shows an abnormal distribution of muscularized tissue. In the wildtype, myotubules radiate laterally from the central tendon. The mutant diaphragm shows myotubule radiation in a more ventral-dorsal pattern. Whole mount diaphragms labeled with neurofilament from E15.5 wildtype (e) and mutant (f) mice. The mutant diaphragm shows a lack of normal phrenic motoneuron branching.

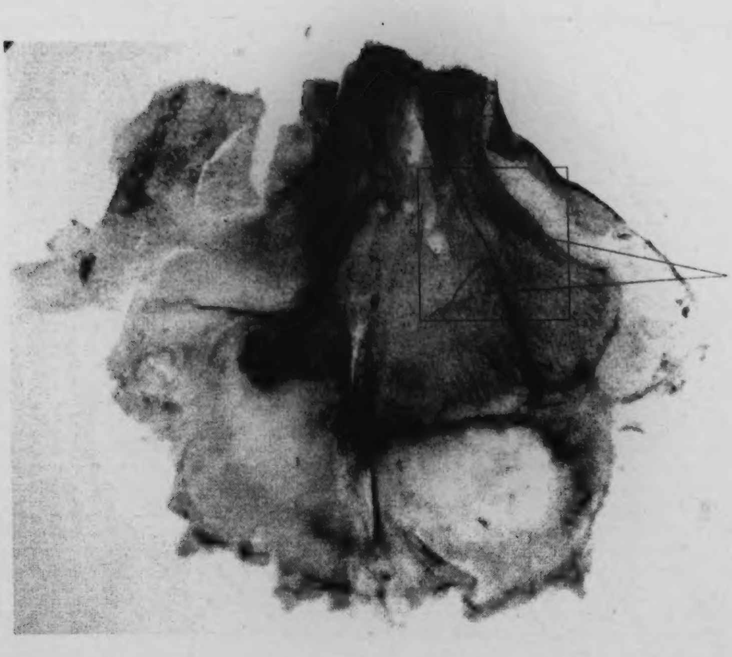

**Figure 4** **The innervation pattern of *Fog2* mutant diaphragms is irregular.**

The pattern of the phrenic nerve branches in the muscular region of an E15.5 mutant diaphragm is irregular (arrows), with fewer branches, as shown by neurofilament immunolabeling. The amuscular membrane that composes the remainder of the diaphragm surface is not innervated.

## ***DISCUSSION***

These mouse models provide intriguing defects in diaphragm development and may provide models to study other rare types of congenital diaphragmatic hernia. These data show that normal diaphragm development is under potentially complex genetic regulation.

### *Implications from the Slit3 diaphragmatic hernia*

The defects seen here initially were thought to be similar to the Morgagni type of CDH. Typical Morgagni hernias are anteromedial or retrosternal and are small triangular defects. It is believed to be a defect between the diaphragm's attachments to the sternum and the costal cartilage. Abdominal contents (bowel, less often the liver, stomach or spleen) may herniate directly through these defects. The majority of hernias through this foramen occur on the right side. These hernias are rare (2-3% of CDH cases seen) and are most often seen later in life, usually upon routine radiographic examination of the chest. It is rarely fatal and does not have a pulmonary component to the pathophysiology like the Bochdalek hernia. In this animal model, the defect is also retrosternal, but unlike a Morgagni defect, which is more frequently located on the right (Stokes, 1991), it is essentially directly medial, centered on the midline of the diaphragm. In the mutant diaphragms, the defect is located in the central tendon, leaving the diaphragm muscle intact and attached to the sternum in normal fashion. This defect actually resembles the central tendon hernia that is a rare type of congenital hernia (1-2% of cases). The central tendon hernia often contains a portion of the liver coming through the central defect (Stokes, 1991). The Morgagni defect is a small triangular defect whereas the mouse defect is oval or circular in shape (Figure 2) and, according to Dr. Alan Flake, a pediatric surgeon in Philadelphia with knowledge of Morgagni hernias, is much greater in its extent. The *Slit3* null mutants are born live, but developed a progressive morbidity that manifested in enlarged chests, atrophic abdomen and respiratory distress, apparently due to problems due to herniation of the abdominal contents into the thoracic cavity. There is some correspondence to the clinical manifestations observed in central tendon hernias, with infants having this defect presenting with varying degrees of respiratory distress.

*Slit*3 is expressed strongly in mesenchymal tissue. It has been shown by others that the *Slit3* mutation decreased the proliferation rate of such cells in the central tendon region of the diaphragm (Yuan et al., 2002). A reduced number of cells could cause the formation of a thin membrane in this region. Since the growing liver provides continuous pressure or tension to the developing membrane, it is conceivable that the membrane could rupture and be penetrated by the liver. The effects of *Slit3* on mesenchymal cell proliferation could be important to normal diaphragm development, but the gene's precise function is yet to be determined.

### *Implications from the Fog2 diaphragmatic hernia*

*Fog2* mutation results in an incompletely muscularized diaphragm with considerable expanses of amuscular membrane separating the abdominal and thoracic compartments. This type of defect does not resemble the more commonly seen types of CDH such as Bochdalek and Morgagni hernias. An examination of the literature found reference to an unusual type of hernia that resembles the characteristics of the defect observed in *Fog2* mutants. It is possible that the defect seen could be described clinically as eventration of the diaphragm (Stokes, 1991). This is characterized as an intact but thin diaphragm resulting from incomplete development of the diaphragm musculature and a paucity of muscle fibers. The defect must encompass an entire hemidiaphragm, rather than one region of a side of the diaphragm, and can be right, left or bilateral. Lung hypoplasia can result if the eventration is severe. All of the diaphragms (4 E15.5, 4 E17.5) examined in this study met these criteria. The extent of the muscularized region of each diaphragm at both ages varied slightly between individual diaphragms (no precise measurements were made), but all showed bilateral muscle defects. Examination of the cardiac system in these mutants showed a variety of defects including septal defects and underdeveloped endocardial cushions and myocardium (Ackerman et al., 2002). These are similar to the effects seen in targeted mutations of the *Fog2* gene. It was observed in mutants that there was significant pulmonary hypoplasia. This led to the designation of the mutation as *lil* (little lung). Previously described mutations in *Fog2* have not reported the diaphragmatic or pulmonary defects. Lung hypoplasia is a common consequence of CDH both clinically and in models. Eventration of the diaphragm can cause interference with lung development (Stokes, 1991), although whether the lung pathology in these mice is a primary effect of the mutation or the result of the diaphragmatic defect is not certain. It is possible that the mutation in *Fog2* directly affects lung development in some way. An interesting observation is that CDH was seen in two cases where there was balanced translocation at the location at 8q22.3, which is the location of the *Fog2* gene. This suggests that *Fog2* may have an important role in diaphragm development and could be involved in CDH formation.

Previously unknown roles for known genes as well as novel genes are being discovered with phenotype-based screening of mutant mice. Through the use of mutagenesis and knockout technology in mice (as well as *Drosophila* and *C. elegans*), information is being gained about the genetic control of development. Correlations of this knowledge with human genes will advance the understanding of human development.

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